Hyaluronan in the neonatal period.

An experimental and clinical study

in asphyxia and infection.

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”As knowledge increases, wonder deepens.”

Charles Morgan

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Papers included in the thesis

I. **Østerholt HCD, Dannevig I, Wyckoff MH, Liao J, Akgul Y, Ramgopal M, Milja, DS, Cheong N, Longoria C, Mahendroo M, Nakstad B, Saugstad OD and Savani RC**

Antioxidant protects against increases in low molecular weight hyaluronan and inflammation in asphyxiated newborn pigs resuscitated with 100% oxygen. PLoS One 2012;7(6):e38839

II. **Østerholt HCD, Lundeland B, Sonerud T, Saugstad OD and Nakstad B**

The impact of hyaluronan on monocyte Toll-like receptor expression in term infant cord blood. Acta Paediatrica. 2012 Jul; 101(7):706-13.

III. **Inchley CS, Østerholt HCD, Sonerud T, Fjærli HO, Nakstad B**

Downregulation of *IL7R*, *CCR7* and *TLR4* in the cord blood of children with respiratory syncytial virus disease. The Journal of Infectious Diseases (In press).

Abbreviations

Base excess (BE)

Bronchoalveolar lavage (BAL)

CC-chemokine receptor 7 (CCR7)

Cluster of differentiation 14, -44, -45 (CD14, CD44, CD45)

Enzyme-linked immunosorent assay (ELISA)

Escherichia coli (E. coli)

European Resuscitation Council (ERC)

Flow cytometry (FCM)

Fluorescein isothiocyanate (FITC)

Forward scatter (FSC)

Glycosaminoglycan (GAG)

Group B Streptococcus (GBS)

High molecular weight hyaluronan (HMW HA)

Human umbilical cord blood (HUCB)

Hyaluronan (HA)

Hyaluronan-mediated motility receptor (RHAMM)

Hyaluronan synthase (HAS)

Interleukin-1 ß, -7, -8 (IL-1 ß, IL-7, IL-8)

Interleukin-7 receptor (IL7R)

Lipopolysaccharide (LPS)

Low molecular weight hyaluronan (LMW HA)

LPS binding protein (LBP)

Matrix metalloproteinase 9 (MMP9)

Median fluorescence intensity (MFI)

Myeloid differentiation primary response protein 88 (MyD88)

Myeloid differentiation protein-2 (MD-2)

N-acetylcysteine (NAC)

3-Nitrotyrosine (3-NT)

Nuclear factor-κB (NF-κB)

Pattern recognition receptor (PRR)

Peridinin chlorophyll protein complex (PerCP)

Phycoerythrin (PE)

Reactive oxygen and nitrogen species (RONS)

Reactive oxygen species (ROS)

Respiratory syncytial virus (RSV)

Reverse transcription polymerase chain reaction (RT-PCR)

Side scatter (SSC)

Superoxide dismutase (SOD)

The International Liaison Committee on Resuscitation (ILCOR)

The receptor intracellular adhesion molecule-1 (ICAM-1)

TIR-associated protein (TIRAP)

TIR-domain-containing adapter-inducing IFN-ß (TRIF)

Toll-like receptor (TLR)

Toll/IL-1 receptor (TIR)

TRIF-related adapter molecule (TRAM)

Tumor necrosis factor-α (TNF-α)

1. What is this thesis about?

This thesis has two main focuses

- To understand the effects of neonatal resuscitation with 100% oxygen on lung hyaluronan (HA). HA is an abundant extracellular matrix component with important biological activities. It is a dynamic molecule that can differently promote or inhibit lung pathology on the basis of its molecular weight and accessibility to various HA-binding proteins. While high molecular weight HA functions to maintain lung structural integrity, low molecular weight HA activates innate inflammatory responses via various signalling pathways. We wanted to study the effects of 100% oxygen resuscitation on HA degradation in the lungs of asphyxiated newborn pigs, and compare the results with other markers of inflammation. Furthermore, we wanted to investigate whether treatment with the antioxidant N-acetylcysteine could decrease fragmentation of HA in the pig lung. We also planned an *in vitro* stimulation of macrophages with HA.

- The other main focus of this thesis is to clarify the biological relevance and activities of HA, its receptors/hyaluronan-binding proteins Toll-like receptors 2 and 4, Cluster of differentiation 44 (CD44) and Hyaluronan-mediated motility receptor (RHAMM) in human umbilical cord blood. Characterization of the distinct inflammatory responses of leukocytes at birth is central to understanding neonatal immunity. Toll-like receptors (TLRs) are key components of the innate immune system as they detect invading pathogens. TLR4 is known to respond to lipopolysaccharide, which is the major component of the outer membrane of Gram-negative bacteria, and is also involved in recognition of respiratory syncytial virus fusion proteins.

  We intended to study the effects of HA on surface expression of TLR2 and TLR4 on monocytes in unstimulated human umbilical cord blood, and also in a situation mimicking Gram-negative sepsis where the blood was stimulated with the lipopolysaccharide. Furthermore we wanted to study whether differences in gene

expression of the hyaluronan-binding proteins/receptors TLR4, CD44 and RHAMM in cord blood leukocytes could predispose to respiratory syncytial virus disease during the first 36 months of life.

In the following “study I” refers to paper I, “study II” refers to paper II and “study III” refers to paper III.

2. Background

2.1 Perinatal asphyxia

Perinatal asphyxia is the single most important cause of brain injury in the newborn. WHO Child Health Epidemiology Reference Group (CHERG) reported that in 2000-2003, asphyxia at birth accounted for 8% of the at that time 10.6 million yearly deaths in children younger than age 5 years (1). Later reports present similar estimates of birth asphyxia (9%) as the cause of deaths (2). Birth asphyxia ranks first among the perinatal insults that cause neurodevelopmental handicap in the newborn, particularly in the full-term baby (3).

Asphyxia features progressive hypoxia (inadequate oxygen supply to the cells and tissues) and hypercapnia (increased levels of carbon dioxide) with a significant metabolic acidosis. Hypoxia is caused by hypoxemia (low oxygen concentration in arterial blood) and/or ischemia (insufficient blood supply) (3). Two main characteristics of perinatal asphyxia are signs of cardiorespiratory and neurological depression, seen on a low Apgar score (≤ 3 at five minutes or later) (4) and metabolic acidosis. A cut off point for defining pathological fetal acidaemia that correlates with an increasing risk of neurological deficit has been found to be an umbilical arterial cord pH of < 7.0 and/or BE < -12 mmol/l or < -16 mmol/l (US and Canada) (5-7).

A hypoxic-ischemic insult starts a cascade of events that ultimately may cause cell death and damage to the central nervous system. The mechanisms for neurodegeneration are not completely understood, but many interconnected pathways are involved in the process of brain injury. These events include depolarization of neuronal membranes, the production of reactive oxygen species leading to oxidative stress, lipid peroxidation, mitochondrial dysfunction leading to the activation of apoptotic pathways, an increase of neurotransmitter release and inhibition of uptake (including the excitatory amino acid glutamate), an increase of intracellular calcium, a decrease of blood flow, and the immunoinflammatory system (8-10). The deprivation of oxygen and glucose play major roles in the initiation of the processes

leading to injury. Moreover, the period of reperfusion has been shown to be the time of occurrence of many of the deleterious consequences of ischemia on brain metabolism and ultimately structure.

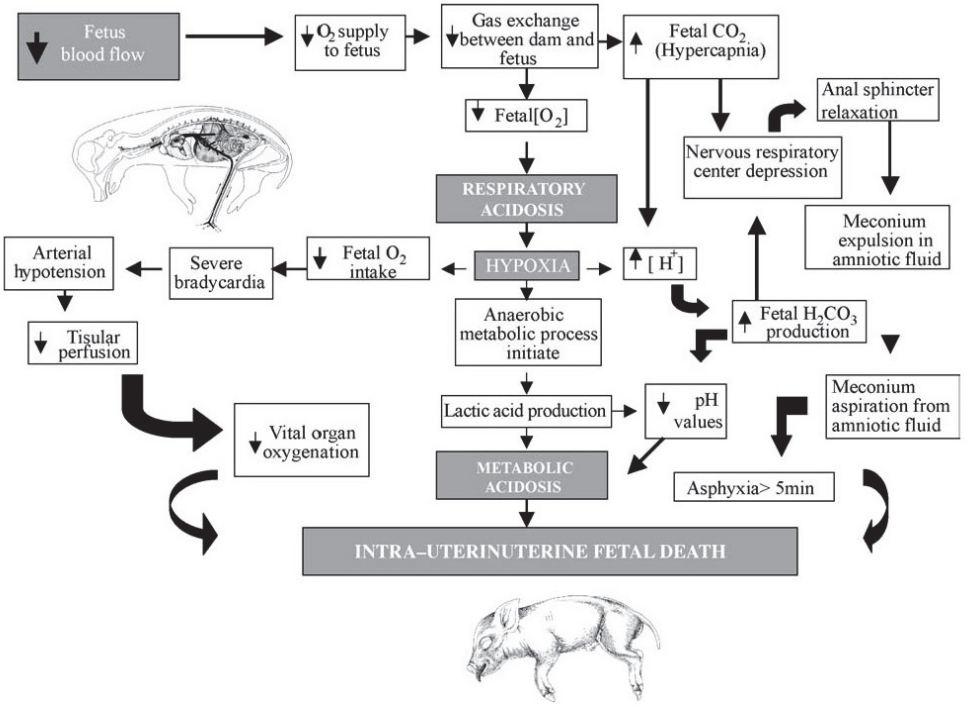

Figure 1. Pathophysiology of the fetal death due to asphyxia in pigs (simplified illustration) (11).

Reprinted from Anim Reprod Sci 2005, Nov; 90(1-2):1-30.

2.2 Resuscitation with O_2

The concept of hypoxia-reoxygenation injury through oxygen free radicals was introduced by Saugstad and Aasen in 1980 (12), and since then, the use of oxygen for resuscitation has been subject to extensive search.

Several studies had been performed on resuscitation of infants with 100% O2 versus room air when we started planning this project in 2006 (13-17). These 5 studies were reviewed by The Cochrane Collaboration in 2005. In the Cochrane review, the authors' stated: "There is insufficient evidence at present on which to recommend a policy of using room air over 100% oxygen, or vice versa, for newborn resuscitation. (…)" (18)

When we initiated our study in 2006, guidelines for resuscitation of neonates were last published in 2005 (19;20). Regarding the use of oxygen the guidelines differed slightly: The International Liaison Committee on Resuscitation (ILCOR) did not specify the concentration of oxygen to be used at initiation of resuscitation. They did however state that there was no evidence to support or refute a change in the oxygen concentration that was initiated, once adequate ventilation was established, and recommended that supplementary oxygen should be considered for infants with persistent central cyanoses (20). The American Heart Association recommended supplementary oxygen to be administered whenever positive-pressure ventilation was indicated, but they did open for the use of room-air if supplementary oxygen was not available (19).

The international resuscitation guidelines are revised every five years. And in their guidelines from 2010, ILCOR now recommends to begin with air rather than 100% oxygen in term and near term infants receiving ventilation at birth (21), whereas the recommendation for preterm babies is still discussed. Since 2010, the European Resuscitation Council (ERC) guidelines advise oxygen saturation (SpO2) targets for the first 10 min of resuscitation after birth. These targets are based on observational studies of healthy term and preterm infants not needing any intervention during their resuscitation (22). During the first 10 min after birth, the SpO2 targets from the ERC

guidelines were advised, i.e. 60%, 70%, 80%, 85%, and 90% at 2, 3, 4, 5, and 10 min after birth, respectively (23).

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Studies from our group had also been performed on newborn pigs. One study from Munkeby et al showed that resuscitation with 100% O_2 increased metalloproteinase MMP-2 activity at both gene and protein levels, accompanied with cerebral leakage of glycerol, presumably triggered by augmented oxidative stress (24). Another study by Solberg et al. showed that supplementary oxygen used for the resuscitation of newborn pigs increased lipid peroxidation in brain cortical neurons, a result that is indicative of oxidative brain damage (25). These findings suggested that resuscitation of asphyxiated newborn pigs with 100% O_2 was detrimental to the piglet brain compared with resuscitation with 21% O_2.

2.3 Free radicals

Free radicals are molecules containing at least one unpaired electron. These highly reactive molecules or chemical species cause oxidative stress, which is defined as “an imbalance between oxidants and antioxidants in favour of the oxidants, potentially leading to damage” (26). Oxidative stress involves generation of RONS (reactive oxygen and nitrogen species, for example peroxynitrite) and ROS (reactive oxygen species, for example superoxide radical). There are numerous sites of oxidant generation, like phagocytic cells (the “respiratory burst”), mitochondrial electron transport, peroxisomal fatty acid metabolism and cytochrome P-450 reactions (27).

Oxidative stress can damage lipids, proteins, enzymes, carbohydrates and DNA in cells and tissues. This can result in membrane damage, fragmentation or random cross linking of molecules like DNA, enzymes and structural proteins and even lead to cell death induced by DNA fragmentation and lipid peroxidation (27). In particular, peroxynitrite causes the nitration of tyrosine residues at the 3-position to produce 3-nitrotyrosine (3-NT) (28).

The brain is very susceptible to oxidative damage due to the fact that the brain contains a high concentration of polyunsaturated fatty acids, which can easily undergo peroxidation, along with regions that are enriched in iron and low levels of endogenous antioxidants (29). The immature brain in particular has a reduced defence to deal with the production of reactive oxygen species, thus it is more susceptible to oxidative stress than the adult brain (30).

During cerebral ischemia, the cut back in oxidative phosphorylation rapidly diminishes reserves of high-energy phosphates. Within a few minutes, considerable amounts of adenosine and hypoxanthine accumulate (31). During reperfusion these metabolic products are metabolised further by xanthine oxidase to produce xanthine and uric acid (32), but this is less likely to occur in the human brain. The breakdown of hypoxanthine by xanthine oxidase in the present of oxygen, produces a flood of superoxide radicals. These can be converted by superoxide dismutase (SOD, one of the body's endogenous antioxidant enzymes) to hydrogen peroxide (33;34).

The activity of xanthine oxidase in the resting brain is very low or minimal (35), but during cerebral ischemia a massive conversion of xanthine dehydrogenase to xanthine oxidase takes place in rats, regulated by the calcium-dependent protease calpain (36). However, in humans (including newborns) xanthine oxidase is probably only present in substantial amounts in the liver and intestine (37-39). In a study in newborn pigs, the release of xanthine oxidase did not seem to correlate with the severity of the histological brain damage after 4 days (40). Thus while xanthine oxidase and oxygen radicals have been implicated in hypoxic-ischemic brain injury in the newborn rat, xanthine oxidase could be less important in the asphyxiated human infant.

2.4 Intervention strategies and the antioxidant N-acetylcysteine (NAC)

After perinatal asphyxia, there is a second phase of injury occurring from approximately 6 to 48 hours after the initial incidence. This phase is characterized by a secondary energy failure (41). The therapeutic window is estimated to be between 2 and 6 hours after the insult (7;42). The goals of management of a newborn infant who has sustained a hypoxic-ischemic insult and is at risk of injury should include early identification of the infant at risk for evolving injury, supportive care to facilitate adequate perfusion and nutrients to the brain, attempts to maintain glucose homeostasis, and consideration of interventions to ameliorate the processes of ongoing brain injury (43).

Several intervention strategies were under investigation when we planned the study resulting in paper I. These included therapeutic hypothermia (44;45), xenon gas (46;47) and allopurinol (48;49). In this thesis I will not describe all the different strategies, but focus on the antioxidant N-acetylcysteine (NAC).

NAC is a well-known antioxidant and free radical scavenging agent, and is able to cross the placenta and the blood-brain barrier (50). It is considered safe during pregnancy (51-54). Glutathione (and the cycle leading to its synthesis), is one of the most important intracellular antioxidant systems (55). Most cells possess enzymes for glutathione synthesis from cysteine, glutamate and glycine, but the capacities vary (55). In preterm infants especially, glutathione synthesis is impaired because of a developmental deficiency of cystathionase activity, leading to extremely low levels of available free cysteine (55;56). L-cysteine is essential for the synthesis of glutathione, and NAC is a source of L-cysteine that bypasses cystathionase.

In the process of preparing paper I, several papers were published describing the use of NAC, showing that administration of NAC reduced cerebral oxidative stress and improved cardiac function and renal perfusion in porcine models of neonatal hypoxia (57-60). In these studies, NAC was given as an intravenous bolus of 150 mg/kg

(57;59;60) or 30 mg/kg (58) at 5 or 10 minutes of reoxygenation, followed by an infusion of 100 mg/kg/h (57)or 20 mg/kg/h (58-60).

2.5 Hyaluronan (HA)

Hyaluronan (HA), also called hyaluronic acid or hyaluronate, is a glycosaminoglycan (GAG). GAGs are large complexes of negatively charged heteropolysaccharide chains. They are generally associated with a small amount of protein, forming proteoglycans. Proteoglycans typically consist of over 95 percent carbohydrate. HA, however, is a unique GAG in that it is non-sulfated and not associated with protein.

HA is a major component of the extracellular matrix (61). Because of its physical properties, HA imparts stiffness and resilience as well as a lubricating quality to many types of connective tissue such as joints. Because of the large number of anionic residues on its surface, HA binds a large amount of water and forms, even at low concentrations, a viscous hydrated gel. If stretched end-to-end, one molecule of HA would be 20 mm long. Overall, however, HA is not a long, rigid rod as is collagen; rather, in solution it behaves as a random coil about 500 nm in diameter.

Over the past decades, evidence has accumulated that HA serves important biological roles beyond its generally accepted function as a structural component of interstitial and connective tissue. For instance, HA has been shown to promote wound healing (62;63), tumor transformation, and metastasis (64-66), as well as cell motility and migration (67-70). Because of its loose, hydrated, porous nature, the HA "coat" bound to cells appears to keep cells apart from one another, giving them the freedom to move about and proliferate. It is also an important mediator of inflammation (71;72). Many of these biological activities are mediated by either the receptor CD44 or the receptor for HA-mediated motility (RHAMM or CD168). RHAMM is expressed in a variety of mammalian cells, including at the apex of ciliated airway cells. HA can also signal through the receptor intracellular adhesion molecule-1 (ICAM-1/CD54), and through Toll-like receptors 2 (TLR2) and 4 (TLR4).

2.5.1 HA and inflammation

HA is an important mediator of inflammation. HA regulation of inflammation is both dose- and molecular size-dependent.

High molecular weight HA (HMW HA, ~1-2 x 10^6 Da) has been reported to inhibit inflammatory cell chemotaxis, phagocytosis and elastase release (73-77). On the other hand, at lower concentrations and at lower molecular weight (LMW HA, < 500-700 kDa), HA promotes monocyte maturation into macrophages as measured by production of insulin-like growth factor-1 (78;79), and HA is greatly increased during inflammatory conditions such as myocardial infarction (80), arthritis (81) and during transplant rejection (82-84). Furthermore, removal of HA with early treatment of myocardial infarction with hyaluronidase results in reduced myocardial fibrosis and infarct size, both in human and in animal studies (85-87).

LMW HA and HA oligosaccharides have been shown to increase gene expression of proinflammatory chemokines and iNOS in macrophage cell lines as well as in alveolar macrophages from injured mice (72) and in microglia (88). Superoxide and peroxynitrite have both been shown to break down HMW HA into LMW HA (89-95). HA is an endogenous TLR4 ligand, and fragments of HA can stimulate chemokine/cytokine production in macrophages by signalling through TLR4, TLR2 and the adaptor molecule MyD88 (96).

In the following, if not otherwise specified, HA denotes both LMW and HMW HA. But in general LMW HA, generated during active inflammation, is an endogenous "danger signal," and HMW HA, predominant in healing or intact tissues, functions in an inverse manner.

2.5.2 HA in the lung

In the lung, HA is mainly located in the peribronchial and interalveolar/perialveolar tissue (97), and is involved in cell-cell interactions, cell-matrix interactions, cell proliferation, tissue hydrodynamics, cell locomotion and inflammation (67;71;98;99). HA also serves a role in airway mucosal host defense. Enzymes are retained at the apical surface of the airway epithelium by binding to surface-associated hyaluronan, providing an apical enzyme pool and protected from ciliary clearance. HA binding to RHAMM stimulates ciliary beating (100).

Interaction of RHAMM with HA is a critical component of the recruitment of inflammatory cells to the lung after injury (71;101). An increased recovery of HA in bronchoalveolar lavage (BAL) has been found in various disease states such as ARDS (102;103).

HA is synthesized by at least three HA synthases (HAS1, HAS2 and HAS3) (104), and HA synthase expression is altered in a number of lung pathologies (105-107). Degradation products of HA is produced under inflammatory conditions, during tissue injury and by oxidative stress (89;91). It is also degraded by hyaluronidases (108). The lower-molecular-weight fragments of HA are implicated in several lung diseases, including asthma, pulmonary hypertension and acute lung injury (109).

Other findings in studies prior to our study in 2006 (resulting in paper I):

- Lung HA concentration had been measured in newborn infants who died within 228 days of birth. The HA concentration in the lung decreased with increasing gestational age. Intrauterine infection was also associated with a significantly higher lung HA concentration (110)
- It had also been shown that HA concentrations in lung extracts of premature monkeys increased with progressively severe RDS (111).

- Lung hyaluronan decreased during group B streptococcal (GBS) pneumonia in neonatal piglets. Atelectasis plus lobar pneumonia further decreased lung HA. It was known that GBS virulence factors include specific secretory enzymes such as hyaluronidase, an enzyme which breaks down HA (112).
- Aerosol administration of HA (molecular weight variable from 400 to 4000 kDa) significantly reduced the bronchial hyperreactivity to muscular exercise in asthma in one study (113). In later studies, aerolized HA has been proposed to have a theurapeutic potential in the treatment of lung diseases like cystic fibrosis (114) and smoke-induced lung injury (115;116).
- Johnsson et al exposed rabbit pups to either room air or hyperoxia (>85%) neonatally. They found that hyperoxia resulted in an increase in lung HA accompanied by an increase in lung water content (117). It had also been shown that hyperoxia caused an increase in lung HA and lung water in neonatal rat pups (118).

In the recent years, the role of HA in lung disease has been summarized in several good reviews (97;109;119;120).

HA and surfactant

HA has been shown to decrease surfactant inactivation in vitro (121). HA has also been shown to reduce surfactant inhibition and improve rat lung function after meconium injury (122). Wang et al published a study where ventilated premature piglets with respiratory distress were given HA-fortified surfactant. They concluded that HA added to 50 mg/kg Curosurf or use of 100 mg/kg Curosurf with or without HA provided the best effects in terms of lung function and reduction of inflammation (123).

2.5.3 HA in the serum and urine

The serum level of HA is regulated by the influx of the polysaccharide from the tissues via lymph and its receptor –mediated clearance by liver endothelial cells. Only 1% of the circulating HA is recovered in urine (124). The half-life of circulating HA in healthy adults is 2,5 – 5,5 min (125). Serum HA is increased in certain liver diseases, especially in patients with cirrhosis, when the clearance is impaired. The serum level also increases in rheumatoid arthritis, in other inflammatory diseases, and it is of special interest that high HA levels in patients with septic conditions is a sign of poor prognosis. Certain tumors, notably Wilms' tumor and mesothelioma, produce factors which activate synthesis of HA and increase its serum level. Rare hereditary diseases with disturbances of HA metabolism and elevated blood levels have also been discovered, e.g. Werner's syndrome and cutaneous hyaluronanosis (126-128).

2.6 Neonatal sepsis

WHO and UNICEF Child Health Epidemiology Reference Group reports that in 2008, neonatal pneumonia or sepsis was estimated to account for 10% of the 8.795 million yearly deaths in children younger than age 5 years worldwide (2).

Trends in sepsis-related neonatal mortality from 1985 to 1998 in the United States have been analyzed: early neonatal mortality from sepsis decreased, on average, from 24.9 to 15.6 deaths per 100 000 live births between the 2 time periods with a greater average annual percent decline from 1995 to 1998 compared with 1985 – 1991. This suggests an association with Group B Streptococcus (GBS) disease prevention efforts and new guidelines concerning intrapartum antibiotic prophylaxis (129;130). In contrast, late neonatal mortality from sepsis increased slightly from 14.8 to 16.2 deaths per 100 000 early neonatal survivors (131). Late-onset sepsis caused by Escherichia coli (E. coli)/gram-negative bacilli has increased in some studies from the U.S. (132;133).

GBS and E. coli are the most common pathogens associated with early onset sepsis in the United States (134;135). Population-based surveillance from 2005 to 2008 conducted through the Active Bacterial Core sites identified 658 cases of early onset sepsis from 2005 to 2008; 72 (11%) were fatal. Overall incidence remained stable during the 3 years at 0.76 to 0.77 cases/1,000 live births. GBS (38%) was the most commonly reported pathogen followed by E. coli (24%) (136). GBS remains the most frequent pathogen in term infants, and E. coli the most significant pathogen in preterm infants (135). In spite of medical advances, E. coli and other gram-negative bacilli remain responsible for significant morbidity and mortality, especially in very low birth weight infants (133;137) (138;139).

Septic shock in the neonatal period has a very poor outcome. An article in Pediatric Critical Care Medicine shows a 28-day mortality of 40%. Adverse outcome at 18 months of corrected age was observed in 24 of 46 infants (52%; death =19, severe sequelae=5) (140).

The increased susceptibility to infection in the neonatal period appears to be due to immaturity of the immune system. The neonatal immune system lacks pre-existing memory and shows decreased Th1-type responses (141-148).

2.7 Toll-like receptors

Toll-like receptors (TLRs) that can be found on monocytes are key components of the innate immune system. They play a central role in the detection of invading pathogens. The german scientist Christiane Nüsslein-Volhard gave name to this pattern recognition receptor (PRR) family (149). Her group found weird-looking larva with under-developed ventral portion of the body when they were studying gene mutations in fruit flies. “Das war ja toll (that is amazing/great)!” was her spontaneous comment when seeing the effect on the larva, and she named the mutated gene Toll (150). Jules Hoffmann’s group described the role of Toll in the immune defence as they found it to have an essential role in the fly's immunity to fungal infection (151). Bruce A. Beutler and colleagues discovered that TLR 4 function as a lipopolysaccharide (LPS) sensing receptor (152). Christiane Nüsslein-Volhard, Bruce A. Beutler and Jules Hoffmann have all been awarded the Nobel Prize in Medicine or Physiology for their work (Nüsslein-Volhard in 1995, Beutler and Hoffmann in 20011).

Of the TLRs, TLR4 is the most characterised, and as written above, it is known to respond to LPS, which is the major component of the outer membrane of Gram-negative bacteria (153). TLR4 is also involved in recognition of respiratory syncytial virus (RSV) fusion proteins (154). TLR2 has the ability to detect various microbial components, such as components of Gram-positive bacteria, mycobacteria and fungi, and this receptor can form a heterodimer with TLR1 or TLR6 (155-158). Polymorphisms in TLR4 may predispose people to develop septic shock with gram-negative microorganisms (159), and to severe RSV bronchiolitis (160), while polymorphisms in TLR2 have been associated with Gram-positive infections in a study of preterm infants (161).

TLR signaling is divided into two distinct signaling pathways, the MyD88-dependent and TRIF-dependent pathway (see figure 2).

In several studies, newborn basal expression of TLR2 and TLR4 has been equivalent to adult levels (145;162). By contrast, others have found significantly lower expression of TLR4 (163) or TLR2 (164) on neonatal monocytes compared to adult levels. Sadeghi et al. did a study on monocytes from preterm infants. They found increasing TLR4 expression with gestational age, and significantly lower expression of the proximal downstream adaptor molecule myeloid differentiation factor 88 (MyD88) in preterm infants (163). A recently published study by Shen et al. shows that the blood monocytes of preterm newborns display rapid increase in TLR2 and TLR4 expression during the first few months of life, whereas LPS-induced cytokine production functionality did not improve in parallel (165). In recent years, an association between TLR polymorphisms and prematurity have been described (166;167), giving new insight into the significance of the human newborn innate immune system.

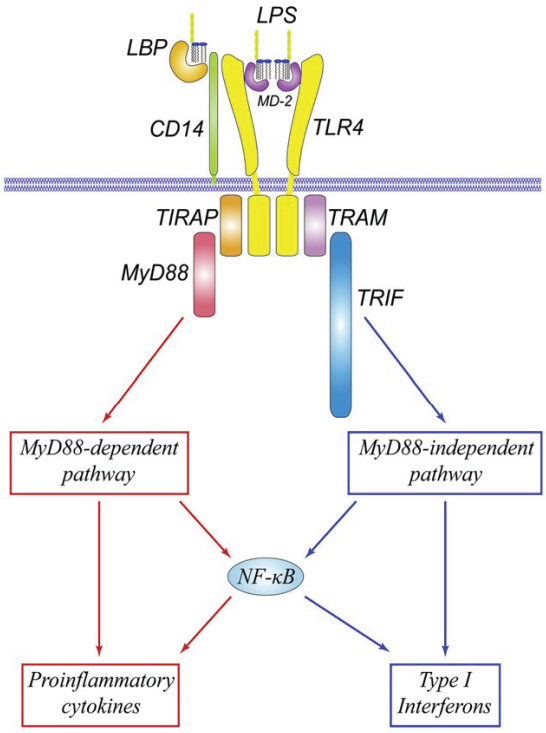

Figure 2. Overview of LPS/TLR4 signalling pathway. LPS recognition is facilitated by LPS binding protein (LBP), and Cluster of differentiation 14 (CD14), and is mediated by a TLR4/ Myeloid differentiation protein-2 (MD-2) receptor complex. LPS/TLR4 signalling can be separated into MyD88-dependent and MyD88-independent pathways, which mediate the activation of proinflammatory cytokine and Type I interferon genes.

TIR: Toll/IL-1 receptor

TIRAP: TIR-associated protein

TRAM: TRIF-related adapter molecule

TRIF: TIR-domain-containing adapter-inducing IFN-ß

2.8 Respiratory syncytial virus (RSV) infection

The fact that RSV responses may be mediated through the monocyte TLR4 receptor inspired our study on susceptibility to RSV infection in infancy (paper III). RSV is a significant human pathogen causing lower respiratory infections among infants and young children worldwide (169). During 1997-2006, an estimated 132,000-172,000 children aged < 5 years were hospitalized for RSV infection annually in the United States (170).

Cellular responses to RSV in vivo and in vitro have been summarized in several good reviews (171-174). In this thesis I am interested in HA and its interactions. Increased tissue degradation of HMW HA is implicated in the pathology of asthma, a condition commonly associated with RSV infection (109). In paper III my focus was therefore on the receptors TLR4, CD44 and RHAMM that regulate HA interactions, and which are likely to be involved in the immune response to RSV (96;100;175). The research group as a whole (paper III) was also interested in other genes that have important functions in both the innate and adaptive immune systems, and that are implicated in the immune response to RSV, and studied gene expression of *IL7R, CCR7* , *CXCL7, IL4, IFNG, CCL3, CXCL11* and *CXCR3*.

Polymorphisms in *TLR4* may predispose children to severe RSV bronchiolitis (160). From the literature it is known that *CD44 variant 6 (CD44v6)* is upregulated in bronchial smooth muscle cells of asthma patients (176). One study by McCurdy et al. showed that RSV proteins colocalized with CD44 and a few other cellular proteins associated with lipid microdomains. This is thought to lead to critical interactions necessary for the formation of filamentous virus particles and syncytium formation (177). It has also been shown that mononuclear leukocytes preferentially bind via CD44 to hyaluronan on human intestinal mucosal smooth muscle cells after RSV infection (175). HA binding to RHAMM expressed at the apex of ciliated airway epithelial cells stimulates ciliary beating, and thus has a role in airway mucosal defense (100). Increased expression of *Has1* and *Has2* in lung tissue was found in a murine model of asthma (106).

3. Aims of the study

Perinatal asphyxia, neonatal sepsis and RSV infection are major causes of morbidity and mortality among infants and toddlers. We wanted to clarify the biological relevance and activities of hyaluronan and its receptors/hyaluronan-binding proteins in relation to these clinical settings, and study the effect of resuscitation with 100% oxygen on hyaluronan in the neonatal lung. We wanted to investigate the following issues:

- Would resuscitation with 100% oxygen versus room air cause more hyaluronan degradation in the neonatal lung? We hypothesized that exposure to 100% O2 during neonatal resuscitation would result in increased peroxynitrite production, fragmentation of HA and an increased inflammatory response (paper I).
- Would antioxidant treatment with NAC prevent a possible degradation of HA?
- We wanted to measure the HA content in serum and BAL after asphyxia, and hypothesized that we would find an increase in HA content in both serum and BAL.
- Would the presence of HMW- or LMW HA alter the monocyte surface expression of TLR2- and TLR4, or the cytokine production, in an *ex vivo* neonatal whole blood model? We hypothesized that the presence of LMW HA would increase the surface expression of TLR4, and that we would find a reduction in TLR4 expression on monocytes (paper II).
- We also aimed to explore whether differences in gene expression of the hyaluronan-binding proteins/receptors *TLR4*, *CD44* and *RHAMM* in cord blood leukocytes could predispose to respiratory syncytial virus disease during the first 36th months of life (paper III).

In figure 3 the TLR4 ligands HA, RSV and LPS are shown, these are in focus in study II and III.

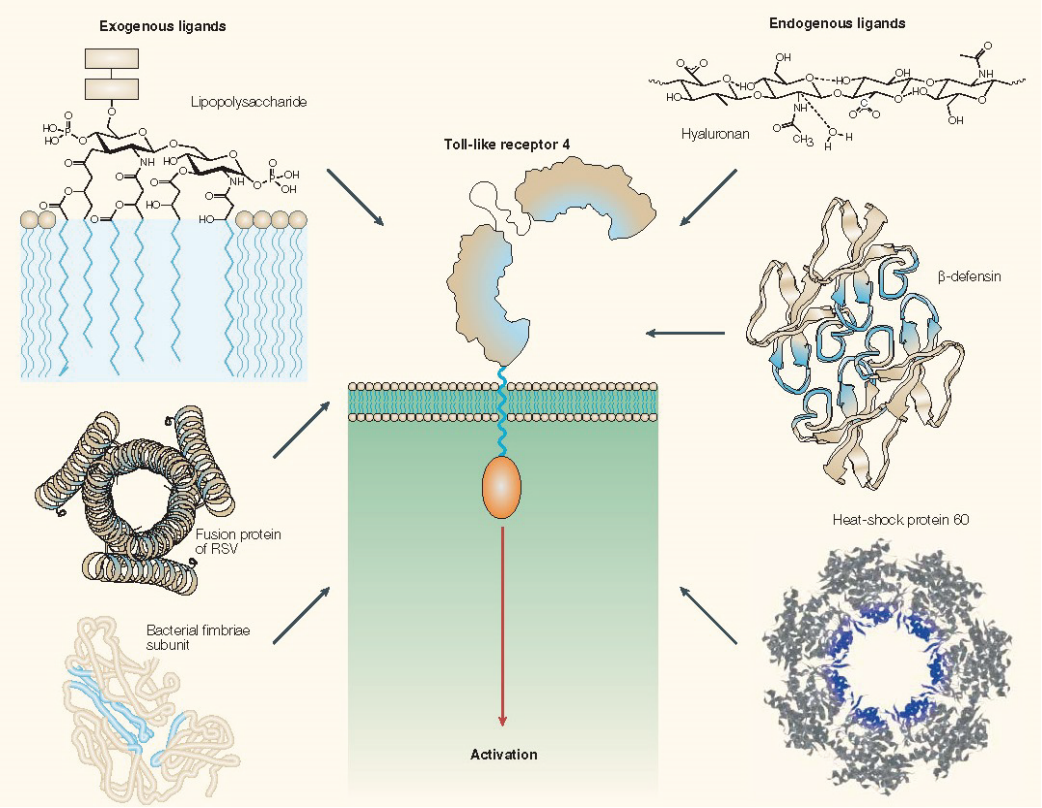

Figure 3. Ligands for TLR4 include both exogenous ligands, such as LPS, the fusion protein of RSV and bacterial fimbriae, and endogenous ligands, such as HA, ß-defensin and heat-shock protein 60 (HSP60). These ligands do not seem to have common structural motifs. Blue colouration indicates hydrophobicity.
Reprinted with permission from Nature Reviews Immunology 4, 469-478 (June 2004) (178)

4. Materials and methods

4.1 Ethical considerations

The animals in study I were cared for and handled in accordance with the European and NIH (National Institutes of Health) Guidelines for Use of Experimental Animals, by certified FELASA (Federation of European Laboratory Animals Science Associations) category C and UT Southwestern researches. The Norwegian Council for Animal Research and the University of Texas Southwestern Medical Center Institutional Animal Care and Utilization Committee approved the experimental protocol.

The Regional Committee for Medical Research Ethics in Norway (Region South East) approved study II and III. In study II written consent for sampling of cord blood was given by the parents after written and verbal information by the first author. In study III oral and written informed consent for further studies were given by the parents both at birth and when the child was hospitalized with acute bronchiolitis during infancy or when attending the follow-up visit at age one year for the controls.

4.2 The animal model (paper I)

It is difficult to study the pathophysiology of neonatal asphyxia in humans. Neonatal hypoxic-ischemic injury has therefore been studied in a variety of animal models. Animal models will always be an approximation of the clinical situation of interest.

The newborn pig model is a popular model due to several reasons. The newborn pigs resemble humans in anatomy and physiology of the cardiovascular system (179;180). In addition to size and morphological characteristics, there are physiologic similarities in the areas of coronary blood flow (181), distribution of the grey/white matter, changes in brain morphology during development (182), growth of the cardiovascular system, and

neonatal pulmonary development (180). Both in human infants and in animal models, the localisation and extent of the injury observed will not only depend on the degree and duration of the insult, but also on the maturity of the brain, lungs and heart (183;184).

We used Noroc pigs in our study. These are a crossbreed between Norwegian Landrace (½), Yorkshire (¼) and Duroc (¼). Newborn pigs (12-36 hours old) were transported from a local farm on the day of the experiment. The pigs were taken from their mother as late as possible on the experiment day to avoid dehydration and stress. They were transported in a warm incubator to avoid hypothermia. Exclusion criteria's were arterial haemoglobin < 5 g/dl, and reduced general condition. Desired weight was 1.5 – 2.0 kg approximately. Total blood volume in a pig is 70 ml/kg body weight and it was possible to withdraw a maximum of 10% blood on the day of the experiment.

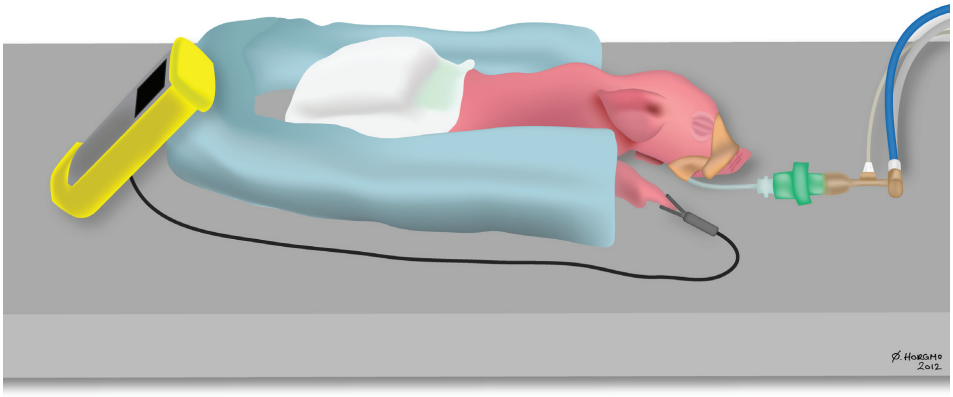

Illustration made by Øystein H. Horgmo, Medical Photography Section, University of Oslo.

4.2.1 Anaesthesia

The animals were weighed and handled when awake, using heated towels and calm surroundings to minimize stress. Anaesthesia was induced by sevoflurane 3-5% (Sevorane, Abbott) mixed with ambient air and oxygen, and was disconnected when an ear vein was cannulated, usually within two-three minutes. The pigs were then given pentobarbital (50 mg/ml) 15-20 mg/kg, midazolam (5mg/ml) 0.5 mg/kg and fentanyl (50 µg/ml) 50 µg/kg intravenously as a bolus injection.

Anaesthesia was maintained by a continuous infusion of fentanyl (50 µg /kg /h) and midazolam (0.25 mg/kg/h IVAC P2000 infusion pump). When necessary, a bolus of fentanyl (50 µg/ml) 50 µg/kg, midazolam (5 mg/ml) 2.5 mg/kg or Pavulon (2mg/ml) 0.1 mg/kg was added. The need for medication was defined as shivering, trigging on the respirator, increased muscle tone and reflexes, increase in blood pressure and/or pulse.

4.2.2 Procedure

The pigs were ventilated with a pressure-controlled ventilator (Babylog 8000+; Drägerwerk, Lübeck, Germany) to achieve normoventilation (arterial carbon dioxide tension ($PaCO_2$) 4.5–6.0 kPa, except during hypoxia) and a tidal volume of 8-12 mL/kg. Inspired fraction of O2 and end-tidal CO2 were monitored continuously (Datex Normocap Oxy; Datex, Helsinki, Finland). They were intubated orally, placed on their back and washed for sterile procedures. Then they got an arterial and a venous catheter. Nutrition and fluid were maintained by giving the pig salidex i.v. (glucose 35 mg/ml, i.e. 3.5%) as a continuous infusion throughout each experiment 10 ml/kg/hour. Mean arterial blood pressure and blood gases were monitored throughout the experiment. Pulse oximetry monitor measured pulse and SaO2. Rectal temperature was maintained between 38.5°C and 40°C with a heating blanket and a radiant heating lamp.

At the end of the experiment, the pigs were given an overdose of 150 mg/kg pentobarbital intravenously. Bronchoalveolar lavage (BAL) and plasma samples were obtained, and

tissues were quickly removed, snap frozen in liquid nitrogen, and stored at -70°C until subsequent analysis.

4.2.3 Experimental group

After intubation and surgical procedures, the pigs had a stabilization period of 60 minutes. Control animals were then euthanized prior to asphyxia. The rest were subjected to global hypoxia and given 8% O_2 in nitrogen until BE < -20 mmol/L and/or MAP 15 mmHg. During hypoxia, CO_2 was added at a $PaCO_2$ 8.0–9.5 kPa, aiming to imitate birth asphyxia. A post asphyxia group was harvested prior to any resuscitation.

The rest of the pigs were resuscitated with either 21% or 100% O_2 for 30 min, and then observed for a further 150 min after resuscitation when all pigs were maintained in 21% O_2. In separate experiments, as described previously (59;60), an additional group of animals exposed to 30 minutes of 100% O_2 resuscitation were treated with a bolus of 150 mg/kg N-acetylcysteine (NAC) starting at the onset of 100% O_2 resuscitation followed by a 20 mg/kg/h infusion for the duration of the experiment.

4.3 Murine macrophage cell line (paper I)

In study I, we also stimulated the murine macrophage cell line RAW264.7 by various concentrations of HA6, the number 6 indicating a six sugar oligosaccharide, for 24 hours. The supernatant was spun to clear cells and stored at -80 °C for later tumor necrosis factor-α (TNF-α) determination. The cells were harvested and Interleukin-1 ß (IL-1 ß) concentrations determined in equal amounts of protein lysate.

4.4 Human umbilical cord blood (paper II and III)

4.4.1 The ex vivo whole blood model (paper II)

Mononuclear phagocytes and neutrophils are crucial cellular components of the innate immune response. Monocytes are mononuclear phagocytes within the blood stream where they circulate for 1 to 3 days (185). Monocytes have retained proliferative capacity and can differentiate into a variety of tissue resident macrophages throughout the body, as well as specialised cells such as dendritic cells and osteoclasts (186;187). We chose this model in order to study monocytes in their natural habitat and to mimic an in vivo condition of acquired E. coli sepsis.

The whole blood model has been described previously (188-190). It is a useful tool for investigating immunomodulating effects on a white blood cell population. Wang et al. showed that leukocyte counts in this model were stable throughout the experimental periode: leukocyte viability exceeded 98% after 14 h of incubation (190).

Human umbilical cord blood (HUCB) for study II was obtained by venopuncture of the umbilical vein immediately after delivery by elective caesarean sections, and collected directly into lithium heparin-containing vacutainers (after informed consent). All specimens were processed within 1 hour. The cord blood was aliquoted into sterile 5 ml polystyrene tubes for different stimulations and incubation times. Some of the blood was not stimulated (referred to as control blood), the rest was incubated with either LPS and/or HA for 3- or 6 hours, as described in paper II. The tubes were incubated in room air at 37 °C, and gently rotated to avoid cell sedimentation. The blood was analysed by flow cytometry and with different immunoassays.

4.4.2 The Akershus Birth Cohort (paper III)

The Akershus Birth Cohort has previously been well described (191). 2108 cord blood samples were collected into Paxgene collection tubes for RNA analysis, into EDTA tubes for later DNA analysis, and into serum tubes for later serum protein analysis. Cross-referencing the cohort with our microbiology database, we identified infants in the cohort who later (before 36 months of age) presented with a positive RSV nasopharyngeal aspirate. Individuals who did not have a positive RSV test were randomly selected from the cohort as controls. We then studied gene expression and performed protein analyses as described in the following sections.

4.5 Laboratory analyses

4.5.1 Immunostaining (paper I)

Immunohistochemistry is a combination of an immunoreaction identifying a specific protein/substance in a selected tissue, and the detection of that reaction using light microscopy. The principle is binding of an antibody to an antigen in the tissue-sample, either direct (using a labelled primary antibody) or indirect (with secondary antibodies, where a primary antibody is first attached to the antigen). The staining can be performed with immunoenzyme-techniques, or immunofluorescence. Immunofluorescence requires specialized microscopes for evaluation.

The immunostaining of lung tissue in paper I was performed on 5 µm paraffin-embedded sections, and indirect dual immunofluorescence for 3-nitrotyrosine (3-NT) and HA was performed as described in the paper. Sections were visualized using fluorescence microscopy, and we used DAPI (a fluorescent stain) for nuclei staining.

4.5.2 Enzyme-linked immunosorent assay (ELISA) (paper I and II)

To measure concentrations of tumor necrosis factor-α (TNF-α) and interleukin-1 ß (IL-1 ß) (paper I, II), and Interleukin-8 (IL-8) and matrix metallopeptidase 9 (MMP9) (paper II), we used commercially available ELISA kits. This is a method that provides a specific and highly sensitive technique for quantification of molecules in a solution, and it is based on the principle of protein binding by specific antibodies combined with an enzyme-induced chromogenic reaction. In short, standards and samples are pipetted into the wells of a microplate precoated with a monoclonal antibody of interest. The protein is bound to the immobilised antibody, and after washing away all unbound substances, a second, enzyme-linked antibody against the protein, making a sandwich complex, is added to the wells. Subsequently, a substrate solution is added, and a colored product is created in proportion to the quantity of protein present in the samples or standards. The

absorbance is measured by a microplate reader, and the protein concentrations are obtained by extrapolation from a standard curve.

ELISA-like assay for HA (paper I)

HA content in BAL and serum was determined by an ELISA-like assay as described previously (101). This ELISA measures the competition of HA present in the sample versus HA coated on a 96-well plate for binding to biotinylated HA-binding protein.

4.5.3 Tissue water content and determination of HA size (paper I)

Wet and dry weights of lung tissue were taken before and after lyophilisation, respectively, and tissue water content was calculated by substracting dry and wet weight ratio from 1. Lyophilized tissues were then treated as described in the Materials and Methods section in paper I, and an agarose gel electrophoretic method for analysis of HA molecular weight distribution was used.

4.5.4 Quantitative reverse transcription polymerase chain reaction (Quantitative RT-PCR) (paper I and III)

This is a technique commonly used in molecular biology to detect RNA expression levels, and is one of many variants of polymerase chain reaction (PCR). Real-time reverse transcription polymerase chain reaction (RT-qPCR) has become the method of choice for the quantification of mRNA. It is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA to cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in real time (192).

4.5.5 Flow cytometry (paper II)

Flow cytometry (FCM) is a means of measuring certain physical and chemical characteristics of cells or particles as they pass in a fluid stream by a beam of laser light. The term "flow cytometry" derives from the measurement (meter) of single cells (cyto) as they flow past a series of detectors. Flow Sorting extends flow cytometry by using electrical or mechanical means to divert and collect cells with one or more measured characteristics falling within a range or ranges of values set by the user. The major applications of FCM include the analysis of cell cycle, apoptosis, necrosis, multicolor analysis, cell sorting, functional analysis and stem cell analysis, etc. We did an immunofluorescent study.

When the cells pass through the laser intercept, they scatter the laser light according to physical characteristics (like size and granulation). In addition, any fluorescent molecule bound to the cell will show fluorescence. The scattered and fluorescent light is collected by lenses, and according to wavelengths routed to the appropriate detectors by combinations of mirrors and optical filters. Voltage pulses are amplified and converted to digital numbers which can be displayed on data plots. The forward scatter channel (FSC) collects light scattered in the forward direction, and its intensity represents an estimate of the cell size. Light scattered approximately at a 90° angel to the exitation line is detected in the side scatter channel (SSC), and the intensity is correlated to the granulation of the cells. By combining FCS and SSC intensity it is possible to separate different cell types, e.g. blood leukocyte subsets.

In study II we used monoclonal antibodies against cluster of differentiation 14, -45 (CD14, CD45), TLR2 and TLR4. CD14 was conjugated with the fluorochromes fluorescein isothiocyanate (FITC), TLR2 and TLR4 with phycoerythrin (PE) and CD45 with peridinin chlorophyll protein complex (PerCP).

We defined the monocytes according to their CD14-staining and side scatter light characteristics. 5000 monocytes were acquired with the help of an electronic gate in each sample. The FACSCanto II flow cytometer (BD Biosciences) equipped with FACS Diva

Software version 6.1.3 (BD Biosciences) was used for the analysis. The median fluorescence intensity (MFI) was recorded and corrected for non-specific antibody binding by subtracting the MFI measured for the matched isotype control sample.

4.5.6 mRNA microarray (paper III)

Microarray analysis can detect the simultaneous expression and interactions of thousands of genes (193).

A microarray works by exploiting the ability of a given mRNA molecule to bind specifically to, or hybridize to, the DNA template from which it originated. By using an array containing many DNA samples, scientists can determine, in a single experiment, the expression levels of hundreds or thousands of genes within a cell by measuring the amount of mRNA bound to each site on the array. With the aid of a computer, the amount of mRNA bound to the spots on the microarray is precisely measured, generating a profile of gene expression in the cell.

In planning study III this technology was used as a hypothesis-generating tool to identify some of the most differentially expressed genes and gene sets, but the HA binding proteins TLR4, CD44 and RHAMM were chosen without using this technology.

4.5.7 Western blot analyses (paper III)

Western blot analysis (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin et al. in 1979 and is now a routine technique for protein analysis. It uses gel electrophoresis to separate proteins by 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are stained with antibodies specific to the target protein.

4.6 Statistical analyses

In paper I differences among groups of animals were analysed using one-way Analysis of Variance (ANOVA). When statistically significant differences were found ($p<0.05$), individual comparisons were made using the Bonferroni/Dunn tests.

In paper II the flow cytometric data were tested for normality using normal probability plots. Thereafter differences between mean values were estimated with 2-tailed Paired-Samples T test. Differences between mean cytokine- and protease concentrations were estimated using the Wilcoxon signed-ranks Test for paired samples (exact method, 2-tailed), since they were not normally distributed. P-values < 0.05 were considered statistically significant.

Paper III. RT-qPCR results were assessed for normal distribution using the Kolmogorov-Smirnova and Shapiro-Wilk tests. Normally distributed data were calibrated to the mean of the control group and analysed using Student's T-test. When data were not normally distributed, they were calibrated against the median of the control group and analysed using the Mann-Whitney or Kruskal-Wallis test, as appropriate. The median difference between control and RSV disease groups was calculated using the Hodges–Lehmann estimator. Mean or median differences were first calculated for $\Delta\Delta$Ct values, and then converted to relative quantities. Subgroup analysis for severity of disease was carried out using One-way ANOVA or the Kruskal-Wallis test, as appropriate, with post-hoc analysis for individual groups vs. control. For protein analysis, a target protein to CD45 ratio was calculated for each sample to correct for variations in sample contamination. Ratios were log transformed to give a normal distribution and analysed using Student's T-test. Correcting for the 11 genes included in the experiment, we considered $p < 0.0045$ to be statistically significant.

5. Summary of results

Paper I

This study included 40 newborn pigs, and there were no significant differences between groups with respect to body weight, age, gender, haemoglobin and baseline hemodynamic- and pulmonary parameters. The 32 animals that were subjected to hypoxia showed similar degrees of compromise at the end of the asphyxia period in both the 21% and 100% O_2 resuscitation groups.

In this study, we found that exposure to 30 minutes of 100% and not 21 % O_2 during resuscitation is associated with increased peroxynitrite and LMW HA content in the lung. An increase in inflammatory cell accumulation and elaboration of TNFα and IL1ß was noted in animals that had LMW HA in the lung. Treatment with NAC was associated with decreased peroxynitrite, preserved HMW HA and less inflammatory cell accumulation and cytokine expression. In cultured murine macrophages, peroxynitrite was able to degrade HMW HA to LMW HA, and oligomeric HA was able to stimulate TNFα and IL1ß production.

At the end of asphyxia, the HA content of BAL decreased and that in the serum increased. BAL HA was significantly increased in animals resuscitated with 100% O_2 and this increase was completely inhibited by treatment with NAC.

Paper II

Concentrations of all cytokines and MMP9 increased significantly when we stimulated the blood with LPS ($P< 0.0005$ for all), indicating an activation of the blood cells in our model.

Our results showed a significant decrease in monocyte TLR4 expression in the presence of HMW HA. Median fluorescence intensity, MFI, was 141 ± 7.3 in the presence of HMW HA, vs. 163 ± 9.8 in control blood (P= 0.019).

There were no significant changes in TLR2 expression on monocytes between control blood and the different stimulations, thus no evidence of HA (neither HMW HA nor LMW HA) having modulating effect on monocyte TLR2 expression.

When the HUCB was incubated with HMW HA for 3- and 6 hours, there were significant increases in concentrations of several proinflammatory cytokines and MMP9, and LMW HA had the same impact at 6 hours. In LPS-stimulated cord blood we measured significant increases in levels of several cytokines and MMP9 (6 h) after exposure to HMW HA, whereas the presence of LMW HA reduced the LPS-induced increments of MMP9 and IL-1ß significantly, while the concentration of TNFα significantly increased.

Paper III

We found downregulation of the cytokine receptors *IL7R* and *CCR7* at birth in the cord blood of children later admitted with respiratory syncytial virus disease. No differences between mild and severe disease groups were detected for these two genes. There was a significant downregulation of *TLR4* in patients later admitted with severe disease, compared to both control group and mild RSV disease group. Subgroup analysis for age did not reveal any significant trends.

The protein results did not statistically confirm the mRNA findings, but the trends supported them.

6. Discussion

6.1 The use of oxygen during resuscitation

In this study, we found an increase in peroxynitrite (a marker of oxidative stress), fragmentation of HA, inflammatory cell accumulation and production of TNFα and IL1ß in the lungs after exposure to 30 minutes of 100% and not 21 % O_2 during resuscitation of newborn pigs. This pro-inflammatory response may cause harm to the lung.

Concerns have been raised regarding potential adverse effects of the use of excessive oxygen, and many studies have addressed this issue. More recently, evidence has accumulated demonstrating that even brief exposure of the lung to hyperoxia is associated with increased mortality, decreased cerebral blood flow and oxidative stress to the kidneys and heart (194-196). The most recent American Heart Association guidelines for neonatal resuscitation emphasize the use of 21 % O_2 for resuscitation of term newborns (197), and a recent report by Vento et al. support a low oxygen strategy for the resuscitation of preterm infants (198)

In the ILCOR guidelines from 2010 it is recommended to begin with air rather than 100% oxygen in term or near term infants receiving resuscitation at birth with positive pressure ventilation. If despite effective ventilation there is no increase in heart rate or if oxygenation, best guided by pulse oximetry, remains unacceptable, a higher concentration of oxygen should be considered. According to the Dawson percentiles median oxygen saturation one minute after birth is 60-70% and increasing to ± 90% after 10 min (22).

In preterm babies of < 32 weeks' gestation, in whom target saturations may not be easily reached in air, blended oxygen and air may be given judiciously, again ideally guided by pulse oximetry. Both hyperoxemia and hypoxemia should be avoided (199).

6.2 Therapeutic potential of N-acetylcysteine (NAC)

In study I treatment with the antioxidant NAC was assosciated with decreased peroxynitrite, preservation of HMW HA and decreased cytokine production. Could this antioxidant therapy have the potential to limit damage after asphyxia? The possible benefit, and the practical approach, of NAC therapy in relations to neonatal resuscitation will need extensive study, and is yet unanswered. In addition, with the use of 21 % O_2 resuscitation, there should be decreased need for the use of antioxidant therapy.

6.3 HA as an endogenous mediator of inflammation, and its possible effect on TLR4 expression

Our study confirms that HA is an important endogenous mediator of inflammation, as shown previously (99). We found a possible effect of HA on TLR4 expression on neonatal monocytes. Campo et al. have found a similar decrease in surface expression of TLR4 in chondrocytes exposed to HMW HA (200). They have also found that adding HA fragments to chondrocytes cultures up-regulated CD44 and TLR4 expression (201), and that LMW HA significantly increased TLR4 expression in both unstimulated- and LPS-stimulated chondrocytes (200), and we therefore hypothesized that the presence of LMW HA would increase the surface expression of TLR4. However, we didn't find any altered expression of TLR4 in the presence of LMW HA. There are limitations of our model, and further research in this field is necessary to clarify the role of different molecular weight HA neonatal infection.

In addition, the possible biological significance of our measured decrease in TLR4 expression in the presence of HMW HA is not known. Regulation of inflammation involves several immune cells and is fine-tuned in order to enhance the host's ability to withstand pathogens in the circulation. An impaired signaling increases the susceptibility of the neonate to infection and invading pathogen. On the other hand, an excessive inflammatory response may be harmful and cause organ damage.

6.4 Can downregulation of leukocyte cell surface receptors at birth predispose to RSV infection in early childhood?

In this study, we hypothesised that differential regulation at birth of cytokine ligands and receptors involved in the immune response to RSV predispose to later RSV disease, including proteins involved in the regulation of lung pathology by hyaluronan, chemoattraction and chemotaxis of myeloid and lymphoid cells, interactions between cells of innate and adaptive immune systems, and regulation of lymphocyte responses

We found downregulation of *IL7R* and *CCR7* in the cord blood of infants later testing positive for respiratory syncytial virus. In addition, those with severe disease had significant downregulation of *TLR4*. This may cause an impaired immune response to RSV, allowing greater viral replication and tissue damage, and thus more severe disease. Further studies are needed in this field.

Interleukin 7 (IL-7) signalling is an important regulator of T-cell survival and homeostasis (202). CC-chemokine receptor 7 (CCR7) regulates migration of dendrocytes and lymphocytes, both to and within lymph nodes, which is an essential part of communication between innate and adaptive immune systems. CCR7 is also involved in the development of antigen tolerance (203).

We know that high molecular weight HA protects against the TLR4 induced inflammatory response to lipopolysaccharide, in a CD44-dependent manner (204). Further that RSV proteins colocalize with CD44, likely leading to critical interactions necessary for the formation of filamentous virus particles and syncytium formation (177). Low molecular weight HA, produced during inflammatory responses in the lung, stimulates cytokine production in macrophages on binding to TLR4 (205). In a mouse model, Zhao et al. showed that on intra-tracheal administration of low-molecular weight HA, TLR4 knockout resulted in a greater inflammatory response, with increased cytokine, chemokine and neutrofil concentrations (206). However, other murine studies indicate that TLR4 knockout protects against inflammatory responses or airways

hyperreactivity (207;208). HA binding to RHAMM expressed on airway epithelial cells cilia stimulates ciliary beating, and thus has a role in airway mucosal defense (100). Polymorphisms in TLR4 may predispose children to severe RSV bronchiolitis (160).

Our study did not show any evidence of predisposal to RSV infection caused by altered expression of *CD44* or *RHAMM* in cord blood. However, in a future study it would be interesting to investigate whether expression of these genes was altered in cord blood of infants who later developed asthma.

6.5 Methodological considerations

Since we have used different models/methods in our studies, I choose to discuss them separately.

Paper I

Some of the differences between human infants and newborn pigs are different biochemical responses, lack of reference values for common functional variables and different maturation at birth. The same NIDCAP-care and type of equipment used in neonatal intensive care units were being used in the experiments. However attractive, the newborn pig model shows large inter-individually variability. Therefore it is important to obtain large enough series for satisfactory statistical analyses. The differences between the pig model and the clinical situation should be kept in mind when results are interpreted. Although the data from our pig models do not necessarily truly mimic human conditions, they can hopefully contribute to the complex understanding of the general mechanisms of injury following neonatal asphyxia.

The use of anaesthesia is a challenge in animal research, as it will affect the results, complicating comparison and interpretation of previous experiments. On the other hand, piglets are easily stressed, and pain and anxiety in the experimental animals will also affect the results by adrenergic stimulation and not reflect the true resting situation. The wellbeing of the animals will always be our first priority. Dose level of anaesthetics for research animals have been developed through veterinary practice and from dose levels used in humans without considering variations between species (180;209). Most of the anaesthetics in the present work are capable of interfering with hemodynamics and cerebral blood flow. However, all animals received the same drugs, so the anaesthesia should have minor influence in the differences found between the groups.

Another drawback of the current study is that the experiments where the animals were given NAC were done separately, and at a later date than the other experiments they were compared to. They were therefore not randomized in the same manner. The intervention

of giving NAC at the onset of resuscitation is also difficult in a clinical situation. In addition, the animals had already undergone fetal to neonatal transition, and were adapted to extra-uterine life when exposed to hypoxia. They were sacrificed at short interval from the injury, and the long term effects of 100% O_2 exposure to the lung, and NAC treatment, were not assessed.

Despite these methodological weaknesses, our study can hopefully provide us with important understanding of mechanisms by which 100% O_2 exposure to the lung causes harm, and stimulate further research in the field.

Paper II

We chose this whole blood model in order to study monocytes in their natural habitat. However, there are variables present in this model that impede the interpretation of results, like the different cell types and proteins in the plasma that bind HA and the intermediates generated by HA action.

It is known that HA fragments signal through CD44 as well as TLR2 and TLR4 to stimulate inflammatory cells. There is also a sequential action of specific proteins of the innate immunity cells which bind and transfer lipopolysaccharide (LPS) to TLR4. In addition, a number of molecules are involved in the intracellular signal transduction. To understand the mechanisms of the cytokine response in our study, efforts to look into other signal transducers and other immune cells are necessary.

HMW HA could be degraded by hyaluronidases in our model. This can explain why both HMW- and LMW HA stimulated production of proinflammatory cytokines and MMP9. Not knowing whether, and to which extent, the HA was degraded during the incubation period is a limitation of our study, along with the number of samples.

In vivo hepatocytes and surrounding connective, vascular and immune cells are important sources of cytokines during acute inflammatory response in severe infections (210).

Pathophysiological events differ from organ to peripheral blood, and from organ to organ (211). Studying the immune response *ex vivo* can not give us a full understanding of the immune response in an organism, but can provide us with useful information and stimulate further research.

Paper III

Our gene expression studies are conducted in cord blood, and one must therefore use caution in drawing conclusions about the pathogenic processes in pulmonary or lymphatic tissue. In addition there is a long time span between the cord blood samples and the later RSV infection, but these studies were undertaken to find out if there is any gene mediated immune response susceptibility for developing RSV disease when exposed to the virus in early childhood. The samples were taken after a stressful experience (birth), which evokes an immune response. In addition, it is likely that individuals in the control group were also exposed to RSV, but they probably had a mild course of disease such as rhinitis, and we believe that the chance of severe RSV disease in the control group was low.

Concerning the protein analysis, the contamination with erythrocytes and plasma in our samples was a drawback. In order to correct for this, we normalized our target protein levels against the level of CD45 in our samples.

It is also important to be aware that the data produced by a Western blot is typically considered to be semi-quantitative. This is because it provides a relative comparison of protein levels, but not an absolute measure of quantity.

7. Conclusions

Compared to 21% O_2 resuscitation, resuscitation with 100% O_2 for 30 minutes resulted in increased fragmentation of HA in the lung of asphyxiated newborn pigs, increased peroxynitrite as a measure of oxidative stress, and increased production of TNF-α and IL-1 ß.

In study I, antioxidant treatment with NAC prevented the degradation of HA, the expression of peroxynitrite and blocked increases in inflammatory cytokines in animals exposed to 100% O_2 during resuscitation.

The HA content of BAL decreased at the end of asphyxia in study I, and the HA content in the serum increased. BAL HA was significantly increased in animals resuscitated with 100% O_2 and this increase was completely inhibited by treatment with NAC.

Our results in study II confirm that hyaluronan affects the neonatal immune response. We found a possible down-modulation of Toll-like receptor 4 exerted by high molecular weight hyaluronan. More studies are needed to validate this finding and the biological significance of these findings requires further clarification.

We found a downregulation of *IL7R* and *CCR7*, but not modulation in gene expression of the HA receptors/hyaluronan-binding proteins *CD44* and *RHAMM*, in the cord blood of infants later (during the first 36 months of life) testing positive for RSV. In addition, those with severe disease had significant downregulation of *TLR4*.

8. Future perspectives

Our studies raise several questions. It would be interesting repeating the experiments in study I with the use of different oxygen concentrations during resuscitation. In addition, the use of NAC as a therapeutic agent needs further investigations. Dose-response studies, evaluation of appropriate time point for administration (administered to the mother during birth, or to the neonate during ongoing resuscitation or afterwards) are questions that are still unanswered. It would also be interesting to assess the long term effects of 100% O_2 exposure to the lung.

We have an ongoing study where we measure serum HA in neonates with sepsis. We aim to clarify the significance of HA during neonatal infection, and its role as a mediator of inflammation. In these neonates we also study surface expression of TLR4 and TLR2 on monocytes, and CD44 on monocytes and lymphocytes. Results will be compared with the same parameters in healthy neonates.

To understand the mechanisms of the cytokine response in study II, efforts to look into other signal transducers and other immune cells are necessary, and requires further research.

Concerning study III, it would be interesting to measure expression of the relevant genes *during* the RSV infection, and to study polymorphisms of these genes. I question whether expression of the genes in our paper are altered in cord blood of infants who later develop asthma, and in other lung pathologies/infections.

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Antioxidant Protects against Increases in Low Molecular Weight Hyaluronan and Inflammation in Asphyxiated Newborn Pigs Resuscitated with 100% Oxygen

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Abstract

Background: Newborn resuscitation with 100% oxygen is associated with oxidative-nitrative stresses and inflammation. The mechanisms are unclear. Hyaluronan (HA) is fragmented to low molecular weight (LMW) by oxidative-nitrative stresses and can promote inflammation. We examined the effects of 100% oxygen resuscitation and treatment with the antioxidant, N-acetylcysteine (NAC), on lung 3-nitrotyrosine (3-NT), LMW HA, inflammation, TNFα and IL1ß in a newborn pig model of resuscitation.

Methods & Principal Findings: Newborn pigs (n = 40) were subjected to severe asphyxia, followed by 30 min ventilation with either 21% or 100% oxygen, and were observed for the subsequent 150 minutes in 21% oxygen. One 100% oxygen group was treated with NAC. Serum, bronchoalveolar lavage (BAL), lung sections, and lung tissue were obtained. Asphyxia resulted in profound hypoxia, hypercarbia and metabolic acidosis. In controls, HA staining was in airway subepithelial matrix and no 3-NT staining was seen. At the end of asphyxia, lavage HA decreased, whereas serum HA increased. At 150 minutes after resuscitation, exposure to 100% oxygen was associated with significantly higher BAL HA, increased 3NT staining, and increased fragmentation of lung HA. Lung neutrophil and macrophage contents, and serum TNFα and IL1ß were higher in animals with LMW than those with HMW HA in the lung. Treatment of 100% oxygen animals with NAC blocked nitrative stress, preserved HMW HA, and decreased inflammation. *In vitro,* peroxynitrite was able to fragment HA, and macrophages stimulated with LMW HA increased TNFα and IL1ß expression.

Conclusions & Significance: Compared to 21%, resuscitation with 100% oxygen resulted in increased peroxynitrite, fragmentation of HA, inflammation, as well as TNFα and IL1ß expression. Antioxidant treatment prevented the expression of peroxynitrite, the degradation of HA, and also blocked increases in inflammation and inflammatory cytokines. These findings provide insight into potential mechanisms by which exposure to hyperoxia results in systemic inflammation.

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Introduction

Worldwide, perinatal asphyxia is the single most important cause of brain injury in the newborn, and has consequences that are potentially devastating and lifelong [1]. Although the hypoxic-ischemic insult starts a cascade of events that ultimately may cause cell death and damage to the central nervous system, a systemic inflammatory response that also affects other organ systems including the heart, lungs, liver and kidneys has been described [2]. Despite earlier reports (dating back to 1897) of similar efficacy of either 100% oxygen (O_2) or room air (21% O_2), which remained largely ignored, neonatal resuscitation, since its inception, has been carried out using 100% O_2 [3]. More recently, evidence has accumulated demonstrating that even brief exposure of the lung to hyperoxia is associated with increased mortality, decreased cerebral blood flow and oxidative stress to the kidneys and heart [4,5,6]. Indeed, the most recent guidelines for neonatal resuscitation emphasize the use of 21% O_2 for resuscitation of term newborns [7], and recent reports support a low oxygen

strategy for the resuscitation of preterm infants [8,9]. Examination of the effects of 100% versus 21% O_2 resuscitation in animal models has been studied most extensively in the pig [10,11]. Indeed, 100% O_2 resuscitation is associated with increased inflammatory markers, decreased anti-oxidant capacity, increased oxidative damage to DNA and proteins, and increased evidence of structural damage to the brain [12,13]. These data suggest that exposure to hyperoxia results in changes that stimulate a systemic inflammatory response that affects multiple organ systems. One potential mechanism could be the formation of reactive oxygen and nitrogen free radicals following exposure to 100% O_2 [14,15].

Three groups of 'reactive species' have been described, namely reactive oxygen species (ROS), which are partially reduced forms of oxygen (for example, superoxide), reactive nitrogen species (RNS), which are partially reduced nitrogen species (for example, nitric oxide (NO)), and the combination of the two, reactive oxygen and nitrogen species (RONS), which are highly reactive and damaging species (for example, peroxynitrite) [14]. Under physiological conditions, ROS and RNS are produced at low fluxes, with minimal production of RONS, and in a localized manner that result in intracellular signaling [14]. However, with increased fluxes of ROS and RNS, the combination of NO with superoxide to form peroxynitrite occurs at a nearly diffusion limited rate, with a dramatic rise in the production of RONS [16]. In contrast to discrete modifications resulting in cellular signaling, the chemical reactivity of RONS results in non-specific modifications that result in cellular injury and death. Importantly, however, the generation of RONS not only generates toxic species but also reduces the availability of ROS and RNS for signaling functions. Therefore the interaction of ROS and RNS is a two-edged sword that produces both a gain of toxicity and a loss of physiological function. The mechanisms of tissue damage with RONS include disruption of cell membranes (lipid peroxidation), mitochondrial injury, modifications of nucleic acids and DNA scission, activation or inactivation of growth factors, apoptosis and the generation of multiple reactive species that cause further damage [15,17]. In particular, peroxynitrite causes the nitration of tyrosine residues at the 3-position to produce 3-nitrotyrosine (3-NT) [18]. Nitration has been shown to result in functional alterations of modified proteins, and has been demonstrated in a wide variety of human diseases and in animal models of tissue injury [19,20,21].

Hyaluronan (HA), a glycosaminoglycan made up of repeating disaccharide units of glucuronic acid and N-acetyl glucosamine, is an early and important mediator of inflammation [22]. An increased recovery of HA in bronchoalveolar lavage (BAL) has been found in various disease states such as sarcoidosis [23], occupational disorders [24] and Acute Respiratory Distress Syndrome (ARDS) [25], and after acute lung injury as with intratracheal bleomycin instillation in rodents [26,27,28]. Further, the increased recovery of HA temporally correlates with an influx of inflammatory cells [29]. HA regulation of inflammation is both dose- and molecular size-dependent. The molecular weight of HA in BAL from injured animals is 200–700 kDa [30], whereas *in situ* lung HA is considerably larger at $>10^6$ Dalton. Low molecular weight HA (LMW HA, $< \sim$500–700 kDa), and HA oligosaccharides (6-30-mer lengths) increase gene expression of proinflammatory chemokines and iNOS in macrophage cell lines as well as in alveolar macrophages from injured rats [31,32]. The changes in localization, content and size of HA during neonatal asphyxia and resuscitation have not been studied previously.

Since superoxide and peroxynitrite cause chemical fragmentation of HA [33,34,35,36,37] and LMW HA can stimulate the inflammatory process [29,38,39], we hypothesized that exposure to 100% O_2 during neonatal resuscitation would result in increased peroxynitrite production, fragmentation of HA and an increased inflammatory response. Using a neonatal pig asphyxia model, we examined the early local effects of resuscitation with 100% O_2 on the lung, and the associated systemic inflammatory response. We here report that exposure of the lung to 100% O_2 was associated with increased 3-NT and decreased HA staining, and fragmentation of HA in the lung. Both TNFα and IL1ß were increased in 100% O_2 exposed animals, and, compared to those with HMW HA content, animals with LMW HA content in the lung had significantly more neutrophils and macrophages, as well as higher plasma concentrations of TNFα and IL1ß. Treatment of 100% O_2-exposed animals with NAC decreased 3NT staining, preserved HMW HA and decreased inflammation and cytokine expression. Furthermore, *in vitro* studies showed that peroxynitrite, and not nitric oxide, fragments HA, and that oligomeric HA stimulates TNFα and IL1ß expression, in a macrophage cell line.

Results

Neonatal Pig Resuscitation Model

There were no significant differences across groups with respect to body weight, age, gender and hemoglobin. Baseline hemodynamic and pulmonary parameters, including blood gases, were also not significantly different between the groups (Table 1). The experimental protocol followed, shown in Figure 1, involved the induction of asphyxia by the administration of 8% O_2. As published by us previously [11], asphyxiated pigs showed profound respiratory/metabolic acidosis, hypoxemia, and hypotension, with similar degrees of compromise at the end of the asphyxia period in both the 21% and 100% O_2 resuscitation groups (Table 2). As expected, the PaO_2 of animals at the end of resuscitation was significantly higher in animals resuscitated in 100% O_2 than those resuscitated in 21% O_2 (49.1±21.1 kPa vs. 13.0±1.9 kPa, $P<0.001$).

Changes in 3-NT and HA Staining with 100% O_2 Resuscitation and NAC Treatment

Formalin-fixed, paraffin-embedded sections of inflated lungs were processed for dual label immunofluorescence using an antibody specific for 3-NT to localize peroxynitrite (red), and a biotinylated HA-binding probe to localize HA (green), with DAPI to label nuclei (blue) as described in Materials and Methods. Images were obtained from both the distal parenchyma and the proximal airway. Control animals had abundant staining for HA in the sub-epithelial matrix around bronchiolar smooth muscle and on the endothelium of blood vessels, with less evident staining in the distal parenchyma and alveoli (Fig. 2). Animals examined 150 minutes after the 30-minutes exposure to 21% O_2 resuscitation had HA staining that was not distinguishable from control animals, and had no staining for 3-NT (Fig. 2). However, 150 minutes after the 30-minute exposure to 100% O_2, little to no HA staining and a significant increase in 3-NT staining was observed throughout the lung (Fig. 2). Interestingly, animals resuscitated with 100% O_2 and treated with NAC had substantially less 3-NT staining, and HA staining that was similar to control animals. These data demonstrate that 3-NT is only evident after hyperoxia exposure and is associated with decreased HA in the lung, and that treatment with the antioxidant decreases 3-NT and preserves HA staining.

Changes in BAL and Serum HA with 100% O_2 Resuscitation and NAC Treatment

We next determined the content of HA in both BAL and serum under the various experimental conditions. Data were compared

Table 1. All pigs were comparable at baseline.

Characteristic	Control (n = 8)	Post-asphyxia (n = 9)	21% O_2 Resuscitation (n = 8)	100% O_2 Resuscitation (n = 8)	100% O_2+ NAC Resuscitation (n = 7)
Weight (g)	1512±189	1585±191	1675±229	1577±274	1309±145
Heart rate (bpm)	149±16	140±13	157±47	147±19	161±20
Mean arterial pressure (mmHg)	55±17	60±14	48±13	50±10	46±6
Arterial pH	7.48±0.09	7.44±0.06	7.47±0.05	7.45±0.10	7.4±0.1
Arterial pCO2 (kPa)	4.9±0.9	5.3±0.6	5.6±0.6	4.8±0.8	3.5±0.4
Arterial pO2 (kPa)	13.5±2.8	13.4±1.9	12.5±1.8	14.3±2.2	11.3±2.4
Base Excess (mmol/L)	3.0±2.8	2.4±5.0	5.7±2.3	0.4±4.5	7.4±3.2
ETCO2 (kPa)	4.1±0.9	4.6±0.5	4.7±0.6	4.0±0.8	5.4±0.7
Lactate (mmol/L)	1.9±0.5	2.0±0.5	2.3±0.8	2.2±0.7	4.4±2.9
Hemoglobin (g)	6.7±1.6	6.9±1.0	6.3±1.5	7.0±0.9	7.4±1.2
Blood Glucose (mmol/L)	6.0±1.5	5.8±1.2	6.3±0.9	5.5±1.0	3.6±1.1

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as a percent of control animals. BAL HA content decreased and serum HA increased with asphyxia (Fig. 3A and B). At 150 minutes after the end of the resuscitation period, animals resuscitated in 100% O_2 had significantly higher BAL HA concentrations than animals resuscitated in 21% O_2 (Fig. 3A). Animals exposed to 100% O_2 and treated with NAC did not show this later increase in BAL HA (Fig. 3A). While serum HA did not show a significant rise at 150 minutes in animals exposed to 100% O_2, treatment with NAC decreased serum HA concentration (Fig. 3B). In order to determine the balance of HA synthesis and enzymatic degradation of HA, we determined the lung mRNA contents for the three HA synthase genes (*has1*, *2* and *3*) as well as two hyaluronidases (*hyal1* and *hyal2*). No significant differences were noted in *has1* and *has3* expression under any condition (data not shown). The expression of *has2* was significantly decreased (Fig. 3C), and the expression of *hyal1* and *hyal2* was significantly increased in animals treated with NAC (Fig. 3D and E), suggesting that the net result would be less HA with anti-oxidant treatment.

Pig lungs were then processed to determine the molecular size of HA as described in Materials and Methods (Fig. 4). Control animals had largely HMW HA, whereas pigs at the end of the asphyxia period had an intermediate size HA. Interestingly, 21% O_2 exposed animals demonstrated HMW HA suggesting increased synthesis, whereas those exposed to 100% O_2 showed almost completely degraded HA (Fig. 4). Interestingly, 100% O_2 exposed animals treated with NAC showed substantial preservation of HMW HA (Fig. 4). Given the decreased *has2* and increased *hyal1/2* expression (Fig. 3), it is most likely that the decreased oxidative and nitrative stress noted with NAC treatment (Fig. 2) prevented the fragmentation of HA. We next determined the proportion of animals with LMW HA in the lung in the various groups studied (Table 3). Interestingly, control animals had almost exclusively HMW HA in the lung, whereas pigs at the end of asphyxia had exclusively LMW HA. Animals resuscitated in 21% O_2 returned to HMW HA whereas those resuscitated in 100% O_2 continued to have LMW HA in the lung. Animals treated with NAC had mostly HMW HA in their lungs. Collectively, these data

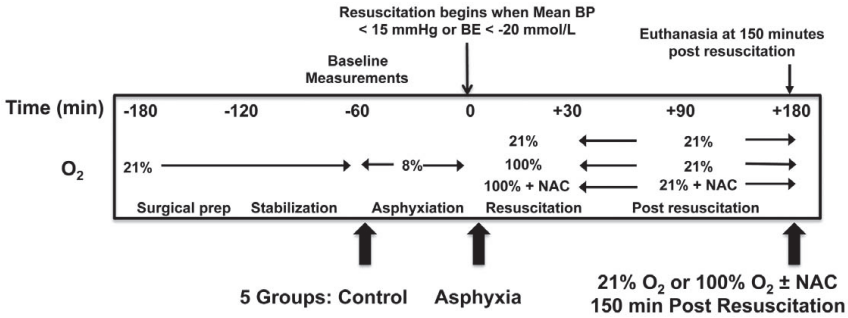


Figure 1. Experimental protocol. After anesthesia, ventilation, and instrumentation followed by 60 min of stabilization, pigs were subjected to asphyxia, followed by either 21% or 100% O_2 resuscitation for 30 min, and then observed for a further 150 min after resuscitation when all animals were maintained in 21% O_2. A separate group of animals resuscitated for 30 minutes with 100% O_2 were treated with the antioxidant N-acetylcysteine (NAC) from the time of the start of resuscitation until the end of the experiment. Samples were obtained from five groups: Control animals were euthanized after undergoing surgical preparation and anesthesia but prior to asphyxia; the asphyxia group was harvested at the end of asphyxia prior to any resuscitation; and three groups of animals resuscitated with either 21% or 100% O_2 or with 100% O_2 and given NAC were harvested at 150 min after respective resuscitation strategies.
doi:10.1371/journal.pone.0038839.g001

Table 2. All pigs were equally asphyxiated.

Characteristic	Control (n = 8)	Post-Asphyxia (n = 9)	21% O_2 Resuscitation (n = 8)	100% O_2 Rususcitation (n = 8)	100% O_2+NAC Rususcitation (n = 7)
Time to asphyxia (min)	–	55±19	63±42	58±25	70±24
Heart rate (bpm)	–	132±33	143±25	160±20	130±25
Mean arterial pressure (mmHg)	–	17±5	15±3	19±4	25±3.2
Arterial pH	–	6.91±0.06	6.92±0.12	6.9±0.09	7.0±0.1
Arterial pCO2 (kPa)	–	9.0±0.3	8.9±1.2	8.9±1.3	8.1±1.3
Arterial pO2 (kPa)	–	4.9±0.4	4.6±0.4	4.6±0.4	4.2±0.3
Lactate (mmol/L)	–	9.4±3.9	11.4±0.9	11.5±3.0	9.7±2.1

doi:10.1371/journal.pone.0038839.t002

suggest that exposure to 100% O_2 was associated with an elevated content of LMW HA, and that antioxidant treatment was associated with a shift to HMW HA.

Increased Lung Neutrophil and Macrophage Contents in Animals with LMW HA are Inhibited by Antioxidant Treatment

We next determined myeloperoxidase (MPO) and N-acetyl glucosaminiadase (NAG) activities of lung as measures of neutrophil and macrophage contents respectively (Fig. 5). Data were segregated according to the molecular size of lung HA and plotted as interquartile ranges. Animals with LMW HA had significantly higher MPO and NAG activities confirming an increased accumulation of neutrophils and macrophages in animals that had LMW HA in the lung (Fig. 5A and B). Animals exposed to 100% O_2 and treated with NAC had significantly lower MPO and NAG activities as compared to animals exposed to 100% O_2 alone (Fig. 5C and D). These data suggest that antioxidant treatment was associated with decreased inflammation after 100% O_2 resuscitation.

Since inflammation is often associated with edema, we also determined whether any differences in water content existed between the various groups. No significant differences in water content were found in any group (data not shown).

Increased Serum TNFα and IL1ß Concentrations in Animals with LMW HA are Inhibited by Antioxidant Treatment

Serum TNFα and IL1ß contents were determined using commercially available ELISA kits. Data were again segregated according to the molecular size of HA in the lung and presented as interquartile ranges. Animals with LMW HA had significantly higher TNFα and IL1ß concentrations (Fig. 6A and B). Treatment of 100% O_2 exposed animals with NAC resulted in significantly lower TNFα and IL1ß concentrations (Fig. 6C and D). Collectively, these data suggest that hyperoxia exposure to the lung results in increased serum TNFα and IL1ß concentrations in association with LMW HA, and that NAC treatment is associated with lower cytokine levels.

Peroxynitrite, but Not Nitric Oxide, Fragments HMW HA to LMW HA In vitro

In order to confirm the specificity of the fragmentation of HA by peroxynitrite, we exposed HMW HA (Healon^{TM}, 1×10^6 Da) to 100 µM 3-morpholinosydnonimine (SIN-1), a compound that spontaneously decomposes to release NO and superoxide to generate peroxynitrite at pH 7.4 (Fig. 7). Exposure of HMW HA to SIN-1 resulted in degradation to LMW HA (300–500 kDa). Exposure to SIN-1 in the presence of superoxide dismutase (SOD) partially protected this degradation, whereas exposure to PA-PANOATE, a pure NO donor, did not degrade Healon^{TM}. In addition, treatment of HMW HA with either *Streptomyces* hyaluronidase at 60°C for 2 hours or sonication for 2 minutes resulted in formation of LMW HA. These data show that superoxide and peroxynitrite, and not nitric oxide itself, fragment HMW to LMW HA.

Oligomeric HA Stimulates TNFα and IL1ß Expression in Macrophages In vitro

In order to determine the effects of LMW HA on TNFα and IL1ß expression, we stimulated the murine macrophage cell line RAW264.7 with a six-sugar length HA oligosaccharide (HA6) that was confirmed to be free of endotoxin, nucleic acid and protein. HA6 (10 µg/ml) significantly stimulated the production of TNFα and IL1ß (Fig. 8). Collectively, these data support the hypothesis that the presence of peroxynitrite fragments HMW to LMW HA and that LMW HA stimulates the production of TNFα and IL1ß in macrophages.

Discussion

In this study, we demonstrate that exposure to 100% and not 21% O_2 during resuscitation is associated with increased peroxynitrite and LMW HA content in the lung. Interestingly, increased inflammatory cell accumulation and elaboration of TNFα and IL1ß was noted in animals that had LMW HA in the lung. Treatment with the antioxidant, N-acetylcysteine (NAC), was associated with decreased peroxynitrite, preserved HMW HA and less inflammatory cell accumulation and cytokine expression. Further, in cultured murine macrophages, peroxynitrite was able to degrade HMW to LMW HA, and oligomeric HA was able to stimulate TNFα and IL1ß production. These data suggest potential mechanisms by which oxygen exposure during resuscitation could fragment HMW to LMW HA and stimulate an inflammatory response.

The use of oxygen in human resuscitation was attempted within 5 years of its discovery in 1772 [3]. Despite later descriptions of the possibility of adequate resuscitation with room air, and pre-clinical studies that demonstrated the potential harmful effects of oxygen radicals, the practice of 100% O_2 resuscitation continued. The Resair2 study, published in 1998 [40], demonstrated that newborn infants could be resuscitated effectively with 21% O_2, and resulted in a faster time to first breath and cry. Further, 21% O_2 resuscitation was not associated with adverse effects in neurodevelopmental

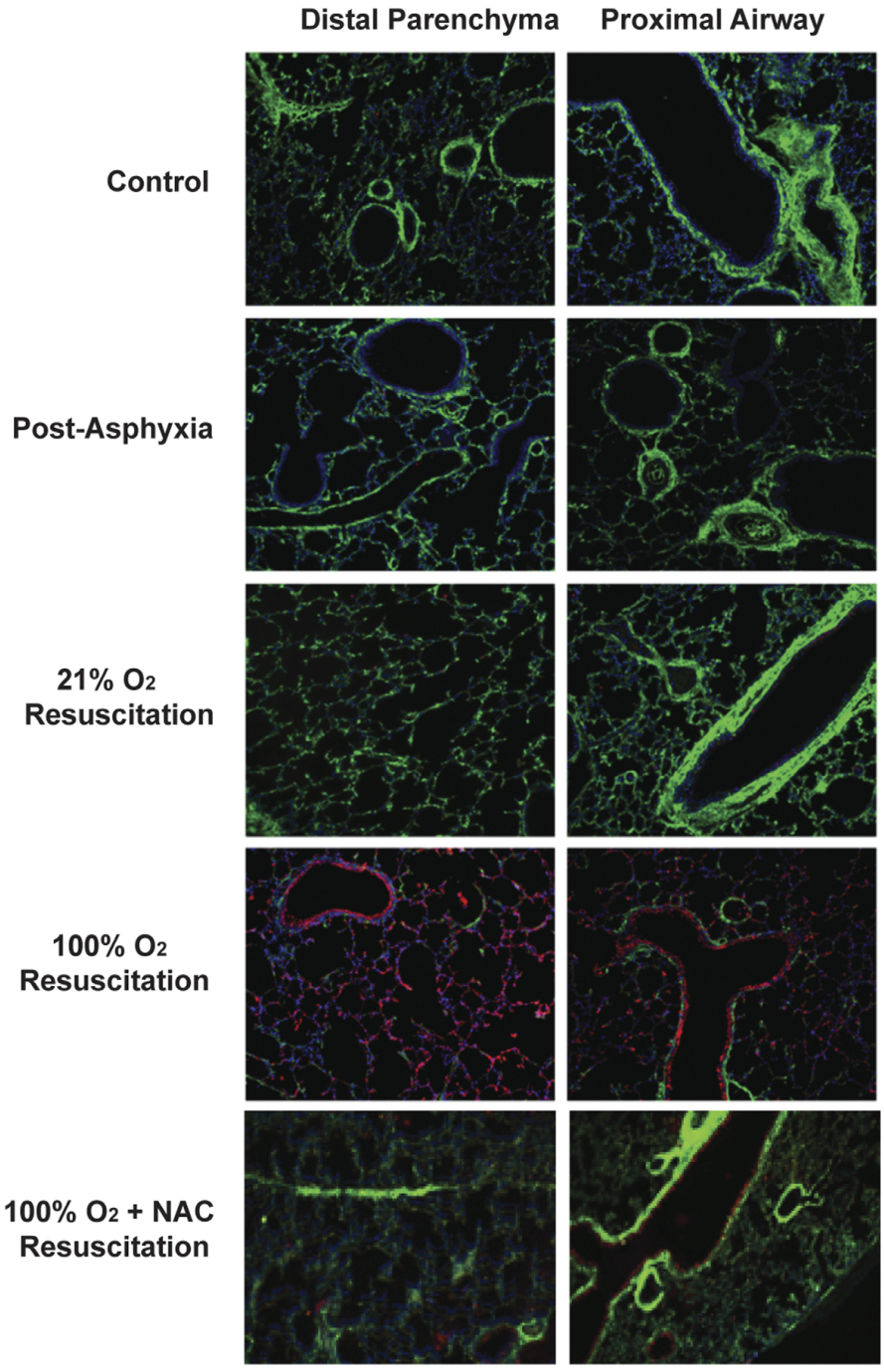

Distal Parenchyma
Proximal Airway
Control
Post-Asphyxia
21% O2 Resuscitation
100% O2 Resuscitation
100% O2 + NAC Resuscitation

Figure 2. Dual immunostaining for 3-nitrotyrosine and HA. Formalin-fixed, paraffin-embedded sections of inflated lungs were processed for dual label immunofluorescence using an antibody specific for 3-NT to localize peroxynitrite (red), and a biotinylated HA-binding probe to localize HA (green), with DAPI to label nuclei (blue). Control animals had abundant staining for HA in the sub-epithelial matrix around bronchiolar smooth muscle and on the endothelium of blood vessels, with less evident staining in the distal parenchyma and alveoli. At the end of asphyxia, there appeared to be a slight, but generalized decrease in HA staining in both the proximal airway as well as the distal parenchyma. Animals examined 150 minutes after 21% O_2 resuscitation had HA staining that was not distinguishable from control animals, and had no staining for 3-NT. However, 150 minutes after 100% O_2 exposure, little to no HA staining and an increase in 3-NT staining was observed throughout the lung. Animals resuscitated with 100% O_2 and given NAC had little 3-NT staining and had HA staining similar to that of animals resuscitated with 21% O_2.
doi:10.1371/journal.pone.0038839.g002

outcome [41]. Multiple other clinical trials have now been conducted that demonstrate that 21% O_2 resuscitation of term infants is as efficient as 100% O_2 and is associated with decreased mortality [4]. However, the mechanisms by which 100% O_2 exposure to the lung causes harm have not been fully examined.

The best-studied model of neonatal resuscitation to date is the newborn pig [10]. The maturation of the pig brain is similar to that of a term human infant, and the newborn pig size allows the use of intensive care equipment used for babies. A number of studies have demonstrated increased accumulation of inflammatory cells in the lung, elevated lung inflammatory markers, and decreased lung compliance with 100% O_2 exposure during resuscitation [42,43]. Additionally, this level of O_2 exposure is associated with decreased anti-oxidant capacity, increased oxidative damage to DNA and proteins, increased apoptosis and evidence of structural damage to the brain [13]. At the same time, it is important to note the limitations of the newborn pig model used in these studies. While useful as a controlled experimental model to determine the mechanisms involved, it does not exactly reproduce the clinical setting. For example, there is no fetal to neonatal transition, the animals receive a long exposure (30 minutes) to 100% O_2, and the animals are sacrificed at very short intervals from the injury.

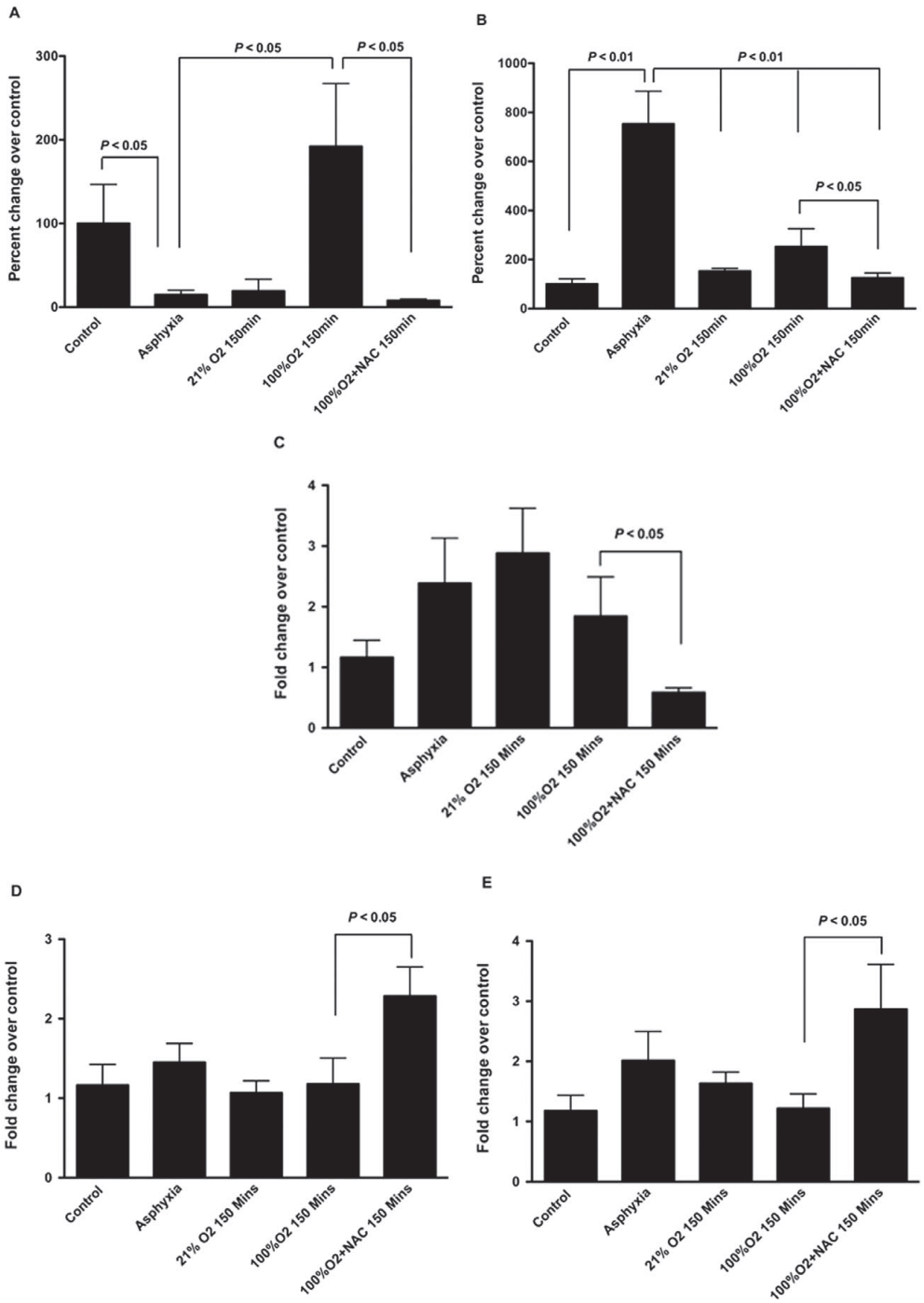
Multiple studies in animal models indicate that superoxide and nitric oxide (NO) participate in hyperoxic lung injury [44,45,46]. High levels of NO, such as those present with inflammation, react with superoxide to yield higher oxides of nitrogen, in particular peroxynitrite [47]. Peroxynitrite, an oxidant capable of damaging alveolar epithelium and pulmonary surfactant [48,49], reacts with proteins to form 3-nitrotyrosine, thereby altering protein function [50,51,52]. Indeed, nitration of myosin light chain 2 in the hearts of asphyxiated pigs is associated with cardiac dysfunction [53]. It is important to remember that exposure to hyperoxia provokes a systemic hyperoxic challenge with effects on multiple organ systems. Indeed, the formation of oxygen and nitrogen free radicals influences the development of pulmonary hypertension [54]. In addition, N-acetyl-glucosaminidase, the macrophage marker used in the current studies, is also found in the liver and kidneys, and is significantly increased in babies with asphyxia that have been resuscitated with 100% O_2 [6]. Cheung et al. have examined the effects of antioxidant treatment in this model of resuscitation. Treatment with NAC, which blocks the production of superoxide and peroxynitrite, results in decreased oxidative stress, improved hemodynamics and tissue perfusion, and decreased platelet aggregation after hypoxia-reoxygenation [55,56,57,58,59,60]. However, the mechanisms by which oxidative and nitrative stresses promote inflammation have not been fully defined.

Hyaluronan (HA) is an early and important mediator of inflammation [22,39]. HA regulation of inflammation is both dose- and molecular size-dependent. HA, at doses of 1 mg/ml or greater, inhibits inflammatory cell chemotaxis [61], phagocytosis and respiratory burst activity [62], as well as elastase release [63]. HA also acts as an anti-inflammatory and anti-fibrotic agent in rheumatoid and osteoarthritis [64], and in repair of tympanic membrane perforations [65]. In addition, HA accelerates cutaneous wound healing [66] and reduces adhesion formation after intra-abdominal surgery [67]. On the other hand, at lower concentrations and at lower molecular weights, HA promotes monocyte maturation into macrophages as measured by production of insulin-like growth factor-1 [68], and HA is greatly increased during inflammatory conditions such as myocardial infarction [69], arthritis [70] and during transplant rejection [71]. Furthermore, removal of HA with early treatment of myocardial infarction with hyaluronidase results in reduced myocardial fibrosis and infarct size [72]. We previously demonstrated that alveolar macrophages from bleomycin-injured animals are more motile than those from control animals and that HA-binding peptide is able to completely inhibit this increased motility [29]. Further, systemic administration of HA-binding peptide to animals prior to injury resulted in decreased macrophage accumulation and fibrosis [29]. These data suggest that HA is upstream of and critical for the inflammatory response to lung injury. HMW HA can be fragmented to LMW HA chemically by exposure to superoxide and peroxynitrite [35,73]. Our findings that treatment with NAC results in decreased 3-NT and preservation of HMW HA, and that the balance of HA synthetic and degrading enzymes favors HA degradation, strongly suggests that HA fragmentation is largely due to chemical rather than enzymatic processes. Thus, treatment with NAC shifts the lung HA environment from a pro-inflammatory to an anti-inflammatory predominance. This is confirmed by our findings that NAC treatment was associated with decreased inflammation and cytokine elaboration.

We also showed that superoxide and peroxynitrite generation by SIN-1 resulted in fragmentation of HMW HA *in vitro*. Importantly, however, a pure nitric oxide donor failed to fragment HA, suggesting that it is the combination of superoxide and nitric oxide that is relevant for the observation. Indeed, administration of high concentrations of NO in conjunction with hyperoxia is harmful to the lung, whereas lower concentrations are beneficial [74]. In addition, studies in extracellular superoxide dismutase (EC-SOD) knockout mice demonstrate that this anti-oxidant enzyme prevents the effects of superoxide and peroxynitrite on fragmentation of HA and limits inflammatory responses to lung injury [75].

The stimulation of cytokine production by HA has been demonstrated previously. However, the mechanisms by which this occurs are unclear. It has been postulated that CD44, a type 1 receptor for HA, complexes with TLR4 to mediate signals that activate the innate immune system [22,39,76,77]. Whether this complex also regulates the production of TNFα and IL1ß after 100% O_2 resuscitation and whether other cytokines are also affected in a similar manner have yet to be investigated.

In summary, we here demonstrate that resuscitation of neonatal pigs with 100% O_2 is associated with increased peroxynitrite, fragmentation of HA and increased TNFα and IL1ß production. Treatment with the antioxidant NAC is associated with decreased peroxynitrite, preservation of HMW HA and decreased cytokine production. The current findings provide proof-of-concept that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Percent change over control
300
200
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100%O2+NAC 150min
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Control
Asphyxia
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Figure 3. Hyaluronan content in bronchoalveolar lavage and serum, and expression of enzymes regulating hyaluronan synthesis and degradation. HA content was determined in BAL (A) and in serum (B). At the end of asphyxia, the HA content of BAL decreased and that in the serum increased. Resuscitation with 21% O_2 did not increase HA either in the BAL or in the serum. However, BAL HA was significantly increased in animals resuscitated with 100% O_2, and this increase was completely inhibited by treatment with NAC (A). The changes in hyaluronan synthase 1 (*has1*), *has2* and *has3*, as well as hyaluronidase 1 (*hyal1*) and 2 (*hyal2*) were determined using quantitative RT-PCR. There were no changes in *has1* and *has 3* expression (data not shown). Expression of *has2* (C) showed a trend to increased expression after asphyxia and 21% O_2 resuscitation. However, treatment with NAC significantly inhibited has2 expression compared to 100% O_2 resuscitation alone (C). The expression of *hyal1* (D) and *hyal2* (E) remained unchanged except that 100% O_2 exposed animals treated with NAC showed significantly increased expression of both hyaluronidases.
doi:10.1371/journal.pone.0038839.g003

antioxidant therapy has the potential to limit damage in situations where 100% O_2 is used during resuscitation. However, the level of O_2 exposure that results in the observations made in this report are yet to be defined in the newborn pig model, and the efficacy of antioxidant therapy during human resuscitation will need extensive study. Indeed, with the use of 21% O_2 resuscitation for term infants, there should be decreased need for the use of antioxidant therapy. The model that we have developed for the mechanisms explored by the current studies is shown in Figure 9. We speculate that limiting the use of 100% O_2 during resuscitation, or blockade of the effects of LMW HA, for example by using HA-binding peptide, will limit the systemic inflammatory response and potentially decrease end organ damage in neonates suffering asphyxia.

Materials and Methods

Ethics Statement

The Department of Comparative Medicine, Oslo University Hospital (Protocol # 37/06) and the University of Texas Southwestern Medical Center Institutional Animal Care and Utilization Committee (Protocol # 2011-0002) approved the experimental protocol. Animals were cared for and handled in accordance with the Norwegian Council for Animal Research, as well as European and NIH Guidelines for Use of Experimental Animals, by certified FELASA (Federation of European Laboratory Animals Science Association) and UT Southwestern researchers.

Surgical Preparation and Anesthesia

Forty newborn Noroc (LYxLD) pigs, 12–36 h of age, Hb ≥5 g/dL, and in good general condition were included in the study. The pigs were anesthetized, orally intubated, ventilated, and surgically prepared as described by Andresen et al. [78]. Briefly, after anesthesia induced by Sevofluran 5% (Sevorane, Abbott), intravenous pentobarbital and fentanyl were administered, and anesthesia was continued with fentanyl and midazolam infusions. A continuous intravenous infusion of 0.7% saline and 1.25% glucose was given throughout the experiment. A tracheotomy was performed and animals were ventilated using a pressure-controlled ventilator to achieve normal ventilation ($PaCO_2$ 4.5–6.0 kPa, O_2 saturations >90% & tidal volume 8–15 ml/kg). The left femoral artery and vein were cannulated with polyethylene catheters. Rectal temperature was maintained between 38.5 and 40°C with a heating blanket and radiant heating lamp.

Experimental Protocol

After 60 min of stabilization, the pigs were subjected to global hypoxia, followed by either 21% or 100% O_2 resuscitation for 30 min, and then observed for a further 150 min after resuscitation when all pigs were maintained in 21% O_2. In separate experiments, as described previously [59,60], an additional group of animals exposed to 30 minutes of 100% O_2 resuscitation were treated with a bolus of 150 mg/kg N-acetylcysteine (NAC) starting at the onset of 100% O_2 resuscitation followed by a 20 mg/kg/h infusion for the duration of the experiment. Samples were obtained from five groups: Control animals were euthanized after

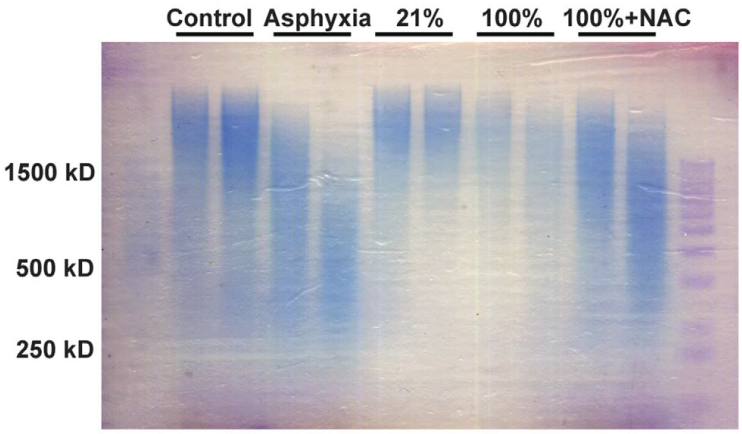

Figure 4. HA size determination. HA size was determined in lung tissue and two representative samples per group are shown in this gel. Control animals had HMW HA and asphyxia was associated with some degradation of HA. Resuscitation with 21% O_2 was associated with HMW HA where as resuscitation with 100% O_2 showed marked degradation of HA. Interestingly, treatment of 100% O_2 resuscitated animals with NAC was associated with a preservation of HMW HA.
doi:10.1371/journal.pone.0038839.g004

Table 3. Proportions of pigs with LMW HA after asphyxia and 21% vs. 100% O_2 resuscitation.

Groups	HMW (n)	LMW (n)	% LMW
Control (n = 8)	7	1	14%
Post-Asphyxia (n = 9)	0	9	100%
21% O_2 Resuscitation (n = 8)	6	2	25%
100% O_2 Resuscitation (n = 8)	3	5	63%
100% O_2+ NAC Resuscitation (n = 7)	5	2	29%

The majority of control animals had HMW HA in the lung. At end of asphyxia, pigs had exclusively LMW HA, which recovered to a preponderance of HMW HA with 21% O_2 resuscitation. Exposure to 100% O_2 resuscitation was associated with a change from HMW to LMW HA. Treatment with NAC showed a protection of HMW HA. These data show that asphyxia and exposure to 100% O_2 is associated with fragmentation of HA in the lung and that antioxidant treatment protects against this degradation *in vivo*.
doi:10.1371/journal.pone.0038839.t003

undergoing surgical preparation and anesthesia but prior to asphyxia; the post asphyxia group was harvested at the end of asphyxia prior to any resuscitation; and three groups of animals were harvested at 150 minutes after the 30 minute exposure to either 21% O_2 resuscitation, 100% O_2 resuscitation, or 100% O_2 resuscitation with NAC treatment.

For asphyxiated groups, hypoxemia was achieved by ventilation with a gas mixture of 8% O_2 in N_2 until either mean arterial blood pressure decreased to 15 mm Hg or base excess (BE) reached -20 mM/L. CO_2 was added during hypoxemia aiming at a $PaCO_2$ of 8.0–9.5 kPa, to imitate perinatal asphyxia. Before resuscitation, hypoxic pigs were divided into experimental groups. Resuscitation was performed for 30 min with 21% or 100% O_2. In separate experiments, a 100% O_2 group was also treated with NAC from the start of resuscitation. After the initial 30 minutes of resuscitation, the pigs all received 21% O_2, were maintained in normocapnia ($PaCO_2$ 4.5–5.5 kPa), and were observed for a further 150 min. Although no animals died during the experiments, two animals observed for 150 minutes following resuscitation (one in the 21% O_2 group and one in the 100% O_2 group) needed short intervals of bag & mask ventilation during this observation period. Three animals that required vasoactive drugs during the studies were excluded from the experimental analysis. At the end of each observation time, the animals were given an overdose of pentobarbital (150 mg/kg IV). Bronchoalveolar lavage and plasma samples were obtained, and tissues were quickly removed, snap frozen in liquid nitrogen, and stored at -70°C until subsequent analysis.

Antibodies & Other Reagents

Polyclonal anti-3-nitrotyrosine antibody was obtained from Cell Signaling Technology (Danvers, MA). Biotinylated HA-Binding Protein (bHABP, Seikagaku Corporation, Tokyo, Japan), a probe that binds all forms of HA six saccharide units or greater, is the biotinylated HA-binding region of aggrecan extracted from nasal bovine cartilage as described by Ripellino et al. [79]. Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Zymed Laboratories (San Francisco, CA). Healon is a pure HMW HA used for ophthalmologic surgery. HA6, a six saccharide HA, was the kind gift of Seikagaku Corporation, and was verified to be free of endotoxin, protein and nucleic acid. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Immunostaining for 3-nitrotyrosine and HA

Indirect dual immunofluorescence for 3-NT and HA was performed on 5 μm paraffin-embedded sections. After paraffin removal and rehydration, endogenous fluorescence was blocked with 0.1% sodium borohydride for 10 minutes followed by incubation with fresh 1M glycine in PBS for 1 hour. Nonspecific binding was blocked by incubation with 100% goat serum for 60 minutes at 4°C. Sections were then incubated with 3-NT antibody (1:500 dilution) overnight at 4°C. Incubations without primary antibody or with pre-immune rabbit IgG were used as negative controls. After washing in PBS with 0.02% Na azide, HA was localized by incubating sections overnight with the biotinylated HA binding region of the aggrecan (bHABP 1:200 dilution; Seikagaku Corporation, Tokyo, Japan). A solution of preincubated HA:b-HABP 3:1 was used as a negative control. Texas Red-conjugated goat anti-rabbit IgG and FITC-conjugated streptavidin (each 1:5,000 dilution) were used as secondary probes, exposed for 3 hours, and DAPI was added to localize nuclei. The slides were then washed and mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL). Labeled sections were visualized using an inverted Nikon TE 100 microscope. Simultaneous wavelength scanning allowed superimposition of fluorescent labeling with FITC and Texas red fluorophores at wavelengths of 488 and 568 nm, respectively. Overlays were accomplished using Metamorph software (Universal Imaging, Downingtown, PA).

ELISA-Like Assay for Hyaluronic Acid (HA)

BAL samples were assayed for HA content by an ELISA-like assay as previously described [29]. This ELISA measures the competition of HA present in the sample verses HA coated on a 96-well plate for binding to a biotinylated HA-binding protein (bHABP Seikagaku, Japan). Briefly, 60 μl of sample or Healon standard (Pharmacia, Sweden) were loaded onto non-fat dry milk (NFDM)-blocked Covalink-NH 96-Microwell plates (Nunc, Fisher Corp.) after overnight protease digestion. After addition of 60 μl bHABP to each well and incubation at 37°C for 1 hour, 100 μl of the sample-bHABP incubation solution were transferred to a HA-coated Covalink-plate and incubated for 1 hour at 37°C to allow to competitive binding (0.2 mg/ml HA, ICN Inc.). HA-binding was detected by an avidin-biotin complex (ABC) reagent (Vectastain) and o-phenylenediamine (Sigma). The change in absorbance at 450 nm after a 15-minute incubation was measured.

Tissue Water Content and Determination of HA Size

Wet and dry weights of lung tissues were taken before and after lyophilization, respectively. Tissue water content was calculated by subtracting dry and wet weight ratio from 1. Lyophilized tissues were digested in 100 mM ammonium acetate with 0.0005% phenol red (pH 7.0) containing 0.25 mg/mL proteinase-K (Roche, Indianapolis, IN) for 4 h at 60°C with occasional vortexing. Proteinase-K was inactivated by boiling, and undigested tissues were pelleted by centrifugation at 16000×g. An aliquot of 100 μl supernatant equal to 5 mg dry weight of digested lung was processed for HA molecular weight determination as described elsewhere with minor modifications [80]. To remove DNA and RNA from tissue extracts, tissues were treated with 3 μl of DNase (Ambion, Austin, Texas) and 3 μl of RNaseA (1.28 mg/ml, Roche, Indianapolis, IN), respectively, for 5 h at 37°C. Samples were boiled to inactivate enzymes and HA was precipitated in 80% ethanol at −20°C overnight. Following centrifugation, pellets were resuspended in 16 μl of Tris-Na Acetate - EDTA, pH.7.9 and 4 μl loading buffer (0.2% Bromophenol Blue, 1 ml TAE buffer and 8.5 ml glycerol). Samples were run on a 1% agarose gel (Seakem HGT Cambrex, Rockland, ME) made in TAE buffer.

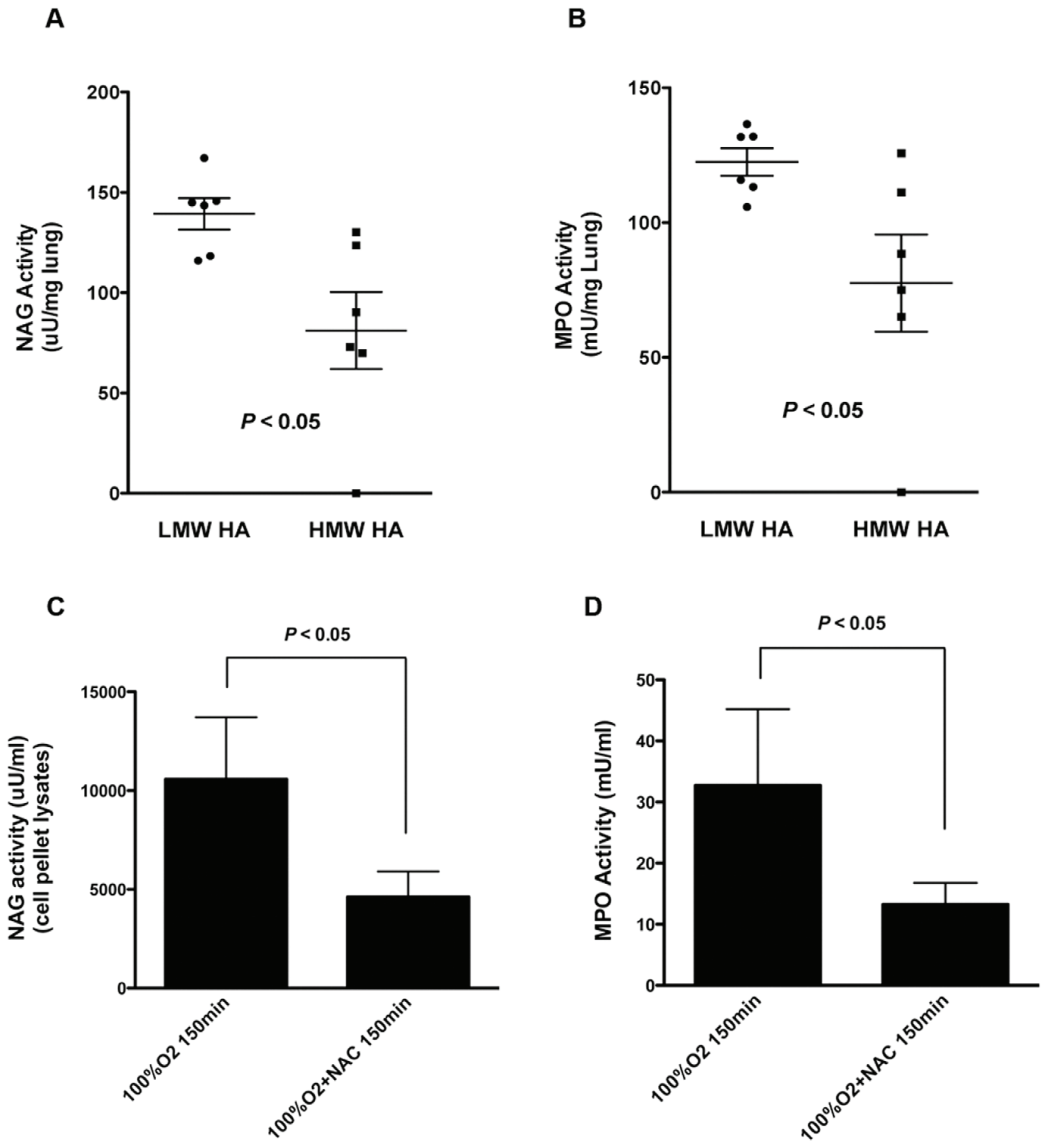

Figure 5. Lung myeloperoxidase and N-acetylglucosaminidase activities as measures of neutrophil and macrophage contents respectively. Macrophage content was determined by NAG activity and neutrophil content was determined by MPO activity as described previously [82] and in Materials and Methods. Data were segregated according to the molecular size of HA found in the lung and plotted as box and whisker plots with outliers shown as additional dots. Both NAG (A) and MPO (B) activities were significantly higher in animals that had LMW HA. Newborn pigs resuscitated with 100% O_2 and treated with NAC had significantly lower NAG (C) and MPO (D) activities than those without NAC treatment, suggesting that antioxidant treatment decreases the inflammatory response to resuscitation with hyperoxia.
doi:10.1371/journal.pone.0038839.g005

The gel was pre-run for ~2 h at 80V prior to loading samples and HA size standards (Hyalose, Oklahoma City, OK). After electrophoresis at 80 V, the gel was equilibrated in water for 48 h followed by incubation in 30% ethanol for 30 min. The gel was then stained with 0.01 mg/ml Stains-All solution (Sigma, St Louis, MO) in 30% ethanol overnight in the dark. Gel was destained in water until bands were visualized before scanning.

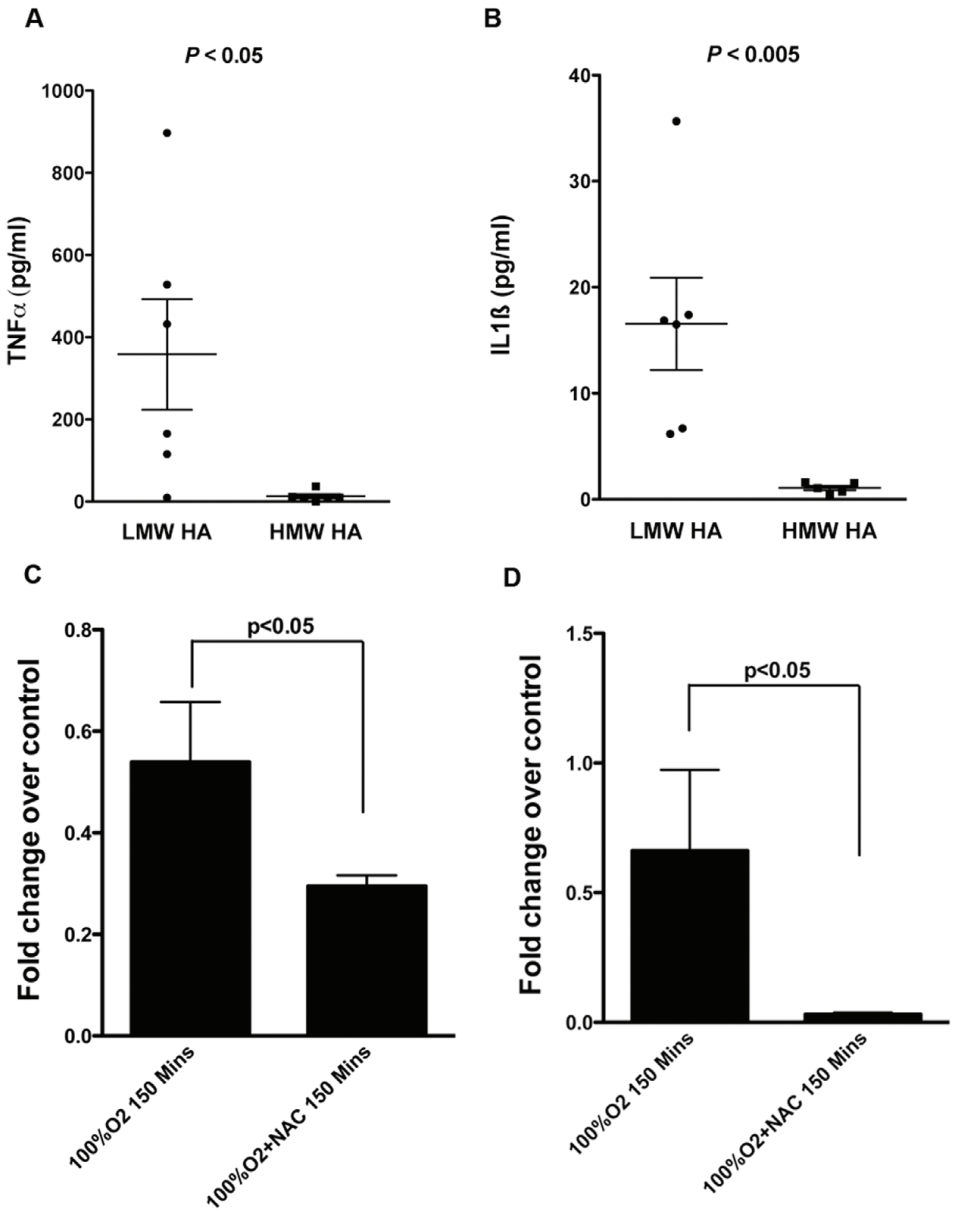

Figure 6. Changes in BAL TNFα and IL1ß concentrations. The BAL contents of TNFα (A) and IL1ß (B) were determined by ELISA were also segregated according to the molecular size of HA. Data are presented as box and whisker plots of 25th and 75th percentiles showing outliers as additional dots. Both inflammatory markers were significantly higher in the animals that had LMW HA in the lung. Newborn pigs resuscitated with 100% O_2 and treated with NAC had significantly lower TNFα (C) and IL1ß (D) than those without NAC treatment, suggesting that antioxidant treatment decreases the inflammatory response to resuscitation with hyperoxia.
doi:10.1371/journal.pone.0038839.g006

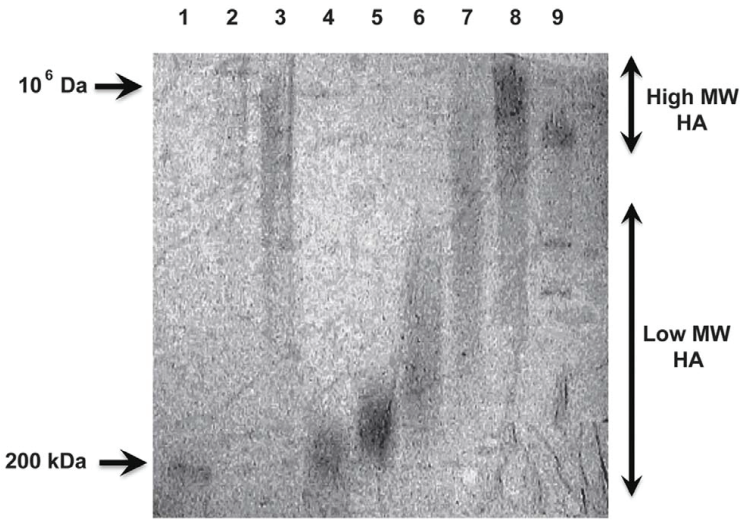

Figure 7. Fragmentation of HA by peroxynitrite and stimulation of TNFα and IL1ß by oligomeric HA in vitro. HMW HA (Healon™, 1×106 Da, Lane 2) was exposed to 100 μM 3-morpholinosydnonimine (SIN-1), a compound that spontaneously releases NO and superoxide to generate peroxynitrite. Exposure of HMW HA to SIN-1 results in degradation to LMW HA (300–500 kDa, Lane 6). Exposure to SIN-1 in the presence of 600 mU/ml superoxide dismutase (SOD) partially protects this degradation (Lane 7). Exposure to 300 μM PAPANOATE, a pure NO donor, does not degrade Healon™ (Lane 8). In addition, treatment of Healon™ with either 1 U/ml Streptomyces hyaluronidase at 60°C for 2 hours (Lane 4) or sonication for 2 minutes (Lane 5) results in formation of LMW HA. Just heating HMW HA at 60°C in the absence of hyaluronidase did not degrade HA in the same manner as in the presence of enzyme (Lane 2). Lane 1 has 200 kDa HA (ICN) and Lane 9 contains Hind III digested DNA makers.
doi:10.1371/journal.pone.0038839.g007

Quantitative RT-PCR

Total RNA was extracted from lung tissues using RNeasy Plus Mini Kit (QIAGEN Inc., Valencia, CA, USA), following the manufacturer's instructions. Extracted RNA concentration of each sample was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription was performed with 1 μg of total RNA in a 20 μl volume using iScript cDNA synthesis kit (BIO-RAD Inc., Hercules, CA, USA). The real time quantitative PCR were

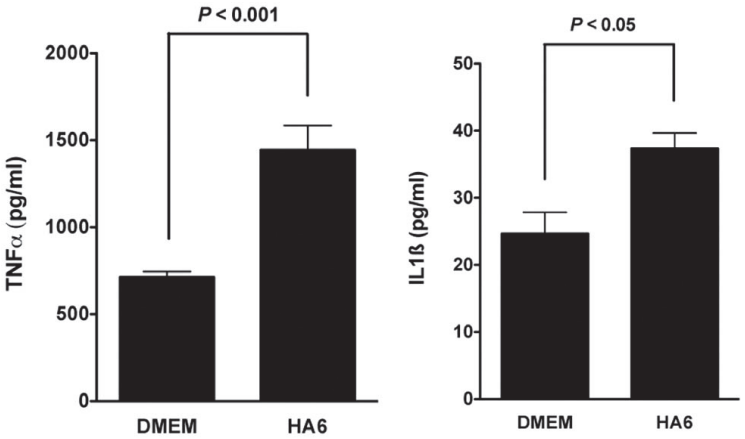

Figure 8. Stimulation of TNFα and IL1ß production by a 6mer HA oligosaccharide. RAW264.7 murine macrophages (1×10^6) were stimulated with a 10 μg/ml of 6-mer HA oligosaccharide *in vitro*. Supernatant IL1ß and TNFα were measured using standard ELISA. HA6 significantly stimulated the expression of both IL1ß and TNFα in the macrophage cell line, thereby confirming that LMW HA can stimulate the expression of inflammatory cytokines in macrophages.
doi:10.1371/journal.pone.0038839.g008

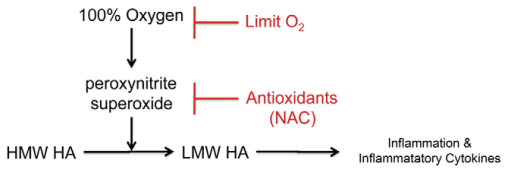

Figure 9. Overall model of the role of oxidative and nitrative stresses and LMW HA in asphyxia and hyperoxia-stimulated inflammation. Exposure to 100% oxygen in asphyxiated newborn pigs results in the production of superoxide and peroxynitrite that cause the fragmentation of HMW to LMW HA. LMW HA, in turn stimulates inflammatory cytokine expression in macrophages and promotes inflammation. Strategies to prevent the formation of LMW HA, such as limiting oxygen exposure or treatment with antioxidants (the current work) will result in decreased inflammation. This model predicts that direct blockade of LMW HA should also achieve the same result.
doi:10.1371/journal.pone.0038839.g009

performed on a 7900 HT fast real-time PCR system (Applied Biosystems, Foster, CA, USA) using 2 µl cDNA, 7 µl diethylpyrocarbonate water, 10 µl SsoFast^{TM} Probes Supermix (BIO-RAD Inc., Hercules, CA, USA) and 1 µl primer of TaqMan Gene Expression Assay. Forty cycles of amplification were performed. Cycle threshold (Ct) values were determined using SDS 2.3 software. The gene of interest was normalized to the Ct value of the endogenous reference gene, 18s rRNA, using the ΔCt method described by Pfaffl [81]. The primer/probe sequences are listed in Table 4.

Myeloperoxidase and N-acetylglucosaminidase Activities

Myeloperoxidase (MPO) and N-acetyl-ß-glucosaminiadase (NAG) activities served as markers of lung neutrophil and macrophage contents respectively, and were determined as previously described [82]. Lung tissue was weighed for normalization of data. For MPO activity, 25 µl of sonicated samples or 4.15 - 83 µU of MPO standards (Sigma, St. Louis, MO) were loaded in 96-well plates. Sixty microliters 0.1M K_2PO_4, 20 µl 0.5% H_2O_2, and 20 µl 1.25 mg/ml O-diansidine (ICN Biomedicals, Irvine, CA) were added to each well. After incubation at room temperature for 15 minutes, the reaction was stopped by the addition of 20 µl 1% sodium azide. The change in absorbance at 450 nm over the 15 minutes provides an index of MPO activity and correlates with neutrophil content. For NAG activity, 20 µl of sonicated sample or 25–400 µU of ß-N-acetylglucosaminidase A standard (Sigma, St. Louis, MO) was loaded in 96-well plates. Ten microliters 0.1% Triton X and 20 µl 15 mM p-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO) were added to each well. After incubation at 37°C for 30 minutes, 200 µl 0.2 M sodium carbonate was added to each well to stop the reaction. The change in absorbance at 405 nm was measured as an index of NAG activity and correlates with macrophage content.

ELISA for TNFα and IL1ß

Serum TNFα and IL1ß concentrations were determined using commercially available ELISA kits (R&D Systems) as per manufacturer's instructions.

In vitro Analysis of HA Fragmentation by Peroxynitrite

In order to confirm superoxide and peroxynitrite fragmentation of HA, we exposed Healon^{TM}, a clinical grade HMW HA product, to various conditions and then examined HA size using gel electrophoresis and staining with *StainsAll*. HMW HA (Healon^{TM}, 1×10^6 Da) was exposed to 100 µM 3-morpholino-sydnonimine (SIN-1), a compound that spontaneously decomposes at pH 7.4 to release NO and superoxide, thereby generating peroxynitrite. HA was also exposed to SIN-1 in the presence of superoxide dismutase (600 U/ml) to decrease superoxide and peroxynitrite. Exposure to PAPANOATE (300 µM), a pure NO donor that does not generate either superoxide or peroxynitrite was used as a control. In addition, Healon^{TM} was treated with either *Streptomyces* hyaluronidase (1 U/ml) at 60°C for 2 hours or sonication for 2 minutes, methods known to fragment HA. In addition to Hyalose HMW and LMW HA markers, Healon^{TM} was used as a marker of HMW HA and HA of molecular size 200 kDa (ICN) was used as a marker for LMW HA.

HA Oligosaccharide Stimulation of RAW264.7 Cells

The murine macrophage cell line RAW264.7 was stimulated by various concentrations of HA6, a six sugar oligosaccharide obtained from Seikagaku Corporation (Tokyo, Japan), which was shown to be free of endotoxin, protein and nucleic acid. Cells (1×10^6) were exposed to 10 µg/ml HA6 for 24 hours. The supernatant was spun to clear cells and stored at −80°C for TNFα determination. The cells were harvested and IL1ß concentrations determined in equal amounts of protein lysate.

Statistical Analysis

At least 7 animals were included in each group. All animal physiology data are presented as mean ± SD. For *in vitro* studies, experiments were repeated at least three times and representative

Table 4. Quantitative real time RT-PCR primers.

Gene of Interest	Forward Primer	Reverse Primer
18s	GAGAAACGGC TACCACATCC	GGACACTCAG CTAAGAGCATCG
IL1ß	AAGGCTCTCCACCTCCTCA	TTGATCCCTAAGGTCACAGGTATCT
TNFα	CCTACTGCACTTCGAGGTTATCG	GGCCAGAGGGTTGA
has1	CTCGGCGACTCGGTGGACTAC	GGGGACCACTGATGCAGGACA
has2	AGCAGCCCATTGAACCAGGGACTTG	AGGGTCGGTGGCGGGCAGTTTCCAAAAC
has3	CCTACTTTGGCTGTGTGAAA	AGGCTGGACATATAGAGAAG
hyal1	CAGTGCCCTAGGTGGACC	CACCCGATCCTTGAGTGAG
hyal2	CGGTATAGGTCTCCCAGTTCTG	CAGGCGCAGTATGAATTTGAG

Forward and reverse primers used for quantitative real time RT-PCR.
doi:10.1371/journal.pone.0038839.t004

data are shown. Results are presented as mean ± SEM. Percent control data were calculated by using the mean of all control animals. Differences among groups were analyzed using one-way Analysis of Variance (ANOVA). When statistically significant differences were found ($p<0.05$), individual comparisons were made using the Bonferroni/Dunn tests.

Author Contributions

Conceived and designed the experiments: HCDO ID MHW BN ODS RCS. Performed the experiments: HCDO ID MHW JL YA MR DSM NC CL. Analyzed the data: HCDO ID MHW JL YA MR DSM NC CL MM BN ODS RCS. Contributed reagents/materials/analysis tools: MM ODS RCS. Wrote the paper: HCDO ID MHW ODS RCS.

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II

III

Brief Report

Downregulation of *IL7R*, *CCR7* and *TLR4* in the cord blood of children with respiratory syncytial virus disease

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Footnotes

The authors declare that there are no commercial or other associations that might pose a conflict of interest.

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Abstract

The association between gene expression at birth of 11 candidate genes with important innate and adaptive immune functions and later RSV disease was investigated. Cord blood was collected from 2108 newborns. 47 were subsequently RSV positive. Gene expression analysis by RT-qPCR was compared to 17 controls. There was downregulation of IL7R (p=0.0001) and CCR7 (p=0.002), and in the severe disease subcategory, downregulation of TLR4 (p=0.003). IL7R and CCR7 facilitate communication between adaptive and innate immune systems. TLR4 activates the innate immune system on RSV exposure. Delayed innate and adaptive immune activation may predispose children to more severe RSV disease.

Key Words:

Respiratory Syncytial Virus; IL7; IL7R; TLR4; CCR7; Children; Gene expression; Immunity; Dendritic cells; Lymphocytes

Background

Respiratory syncytial virus (RSV) causes yearly epidemics of bronchiolitis and viral pneumonia in the pediatric population. About 2/3 of all children are exposed the first year of life, and almost all by the age of 2 years [1]. Most have mild upper airways disease. 22 - 31/1000 require hospital admission [1, 2], but have usually had a normal gestation and birth, and have otherwise appeared healthy. Why so few exposed to RSV require hospital admission is a focus of on-going research.

Cellular responses to RSV are summarized in several reviews [3, 4]. Pulmonary dendrocytes are key innate immune cells of the respiratory epithelium that recognize RSV via Toll-like receptor 4 (TLR4). Dendrocytes then recruit granulocytes and monocytes, and migrate to regional lymph nodes, where they present RSV-antigen to lymphocytes, mobilizing the adaptive immune system.

In this study, we hypothesize that differential regulation at birth of ligands and receptors involved in the immune response to RSV predisposes to later RSV disease, including proteins involved in the regulation of lung pathology by hyaluronan, chemoattraction and chemotaxis of myeloid and lymphoid cells, interactions between cells of innate and adaptive immune systems, and regulation of lymphocyte responses.

Methods

The Akershus Birth Cohort is described in our previous papers [5, 6]. From 3500 births, we collected 2108 cord blood samples into PAXgene collection tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) and EDTA tubes.

We cross-referenced the microbiology database with the birth cohort to identify participants who later had a positive RSV test. Patients were included if RSV positive before 36 months of age. 17 Individuals not tested for RSV were randomly selected from the cohort as controls.

Exclusion criteria:

Cases and controls were assessed for the following exclusion criteria September 2009, at age 5 – 6 years: i) conditions known to predispose to severe RSV disease: congenital heart disease, gestational age at birth < 34 weeks; chronic airways diseases (eg: bronchopulmonary dysplasia, asthma, recurrent lower airways infections congenital airways anomalies), or Down's syndrome. ii) Conditions affecting gene regulation at birth: small for gestational age, perinatal infection, metabolic or neurological disease. iii) Controls who had moved from our hospital population area, or who were admitted to hospital with other respiratory disease.

Disease categorization:

Medical records were assessed retrospectively. Criteria for severe RSV disease were: mechanical respiratory support; apnea related to respiratory exhaustion; supplementary oxygen requirement; intravenous or nasogastric fluid; a significant level of respiratory distress, as documented by the pediatrician. Criteria for mild RSV disease were: tested for RSV by their GP, but not sent to hospital for admission; sent home from hospital without admission; only upper airways disease; mild or no respiratory distress, as assessed by the pediatrician.

RT-qPCR analysis:

Total RNA was isolated from cord blood samples and prepared as described in our previous paper [5]. Gene expression was quantified by RT-qPCR of mRNA for *IL7RA, CCR7, CXCL7, TLR4, IL4, IFNG, CCL3, CXCL11, CXCR3, CD44* and *RHAMM*. Please see the supplementary material for a detailed description of the RT-qPCR analysis.

Protein analysis:

Genes significantly regulated in the RT-qPCR were selected for protein analysis. Because these genes encode cell membrane-bound proteins, EDTA samples were analyzed. 10 samples from each group (control, mild, and severe disease), representative of the interquartile range for mRNA expression for each target protein, were analyzed. Protein extraction and Western blot analysis was carried out as described previously [5].

In 2003, cell-layer separation resulted in contamination with erythrocytes and plasma in our leukocyte samples. To correct for this, target proteins were normalized against the leukocyte-specific surface protein CD45 in our samples [7]. To verify leukocyte specificity, new cord blood samples were collected in 2011, and leukocyte, erythrocyte and plasma layers carefully separated and analyzed by Western blotting. Please see the supplementary material for a detailed description of the protein analysis.

Statistical analysis:

RT-qPCR data was assessed for normal distribution using the Kolmogorov-Smirnova and Shapiro-Wilk tests. Normally and non-normally distributed data were analyzed using Student's T-test and the Mann-Whitney test, respectively. The difference in medians was calculated using the Hodges–Lehmann estimator. Differences in mean or

median were first calculated for ΔΔCt values, and then converted to relative quantities. Subgroup analysis for severity of disease utilized one-way ANOVA or the Kruskal-Wallis test. For each sample, target protein to CD45 ratio was calculated, log transformed and analyzed using Student's T-test. A power analysis of previous results indicated a need for 11 individuals in each group for a power of 80% in the mRNA analysis. We included 17 in the control group. Statistical analyses were carried out using Prism 5 (GraphPad Software inc), SPSS 19 and Minitab 15.0 statistical software. Using Bonferroni's correction, $p < 0.0045$ was considered significant.

Ethical issues: Regional Ethics Committee approval was obtained before data collection began. A guardian gave informed, written consent before study inclusion.

Results:

Of 2108 children, 70 were RSV positive before age 3 years; 3 had asthma, 1 had repeated pneumonia, 1 had cleft palate and 1 had Down's syndrome. 64 were eligible for inclusion; 47 had sufficient RNA for analysis. The mean age on positive RSV testing was 7.7 months (SD 9.4 months); median age 3 months (IQR 2 – 8 months); 37 were aged < 12 months, and 10 aged 12 months to 35 months. 25 had severe, and 22 mild disease. Birth, clinical and intervention data are presented in table 1. 8 RSV positive patients were not referred to hospital and were therefore classified with mild disease.

Gene Expression: When considering all children with confirmed RSV infection, there was a significant downregulation of both *IL7R* (relative expression 69%; 95% CI 58 – 83%; $p = 0.0001$) and *CCR7* (relative expression 78%; 95% CI 67 – 91%; $p = 0.002$) in the RT-qPCR analysis. (Figure 1A).

On subgroup analysis for disease severity (figure 1B), *IL7R* and *CCR7* had a significant one-way ANOVA (p = 0.0006 and p=0.004, respectively) and *TLR4* tended to significance (p=0.02). Post-hoc analysis revealed significant downregulation of *TLR4* amongst children with severe RSV disease, compared to controls (relative expression 64%; 95% CI 48 – 85%; p = 0.003).

Protein analysis: Western blot analysis to verify CD45 specificity is presented in the supplementary material (figure S1). We can correct for erythrocyte, but not plasma contamination, due to non-specific anti-CD45 antibody binding to plasma proteins. Plasma contamination is likely to be evenly distributed between groups, reducing leukocyte-protein concentration, but not the ratio of means. However, intra-group variation is likely, reducing statistical power.

IL7R, *CCR7* and *TLR4* were selected for protein analysis. Figure 1C presents CD45 ratios for controls and cases. There were no significant associations but there are obvious trends that support our mRNA findings.

Discussion:

In this nested case-control study, we find downregulation of *IL7R* and *CCR7* at birth in the cord blood of children who are later RSV positive. There was significant downregulation of TLR4 in patients later admitted with severe disease. For several genes a large variance meant a lack of power to detect clinically interesting differences. This was particularly relevant for *CXCR3; IFNG; IL4; RHAMM* and *CXCL11*.

Whilst protein results do not statistically confirm our mRNA findings, the trends do support them. The Western blot experiment may have lacked power to detect

significant differences. We cannot exclude altered protein expression due to posttranscriptional regulation of mRNA by microRNA or other mechanisms.

Control individuals were probably exposed to RSV. We have no information about their clinical symptoms other than that they did not present to pediatric units in our region, and that their GP did not send a nasopharyngeal aspirate for RSV detection. Given our knowledge of our catchment population, public expectations of Norwegian health services, and the tendency for GPs in our area to refer younger children for assessment by a pediatrician, we believe it probable that control individuals had a mild course of RSV disease such as rhinitis, and that the chance of severe RSV disease in the control group was low. However, we cannot exclude overlap between mild and control groups. Disease severity subgroup allocation may be confounded by retrospective collection of clinical data, leading to erroneous classification. This will not have affected the results for *IL7R* or *CCR7,* where mild and severe groups were similar, but may affect results for *TLR4.*

IL7RA is in peripheral blood expressed by mature T-lymphocytes, closely determining cellular responses to interleukin-7 (IL7). IL7 signaling is an important regulator of T-cell survival and homeostasis, preventing apoptosis, promoting T-cell proliferation in some conditions, maintaining peripheral T-lymphocyte levels during immune responses, and possibly regulating effects of T-cell receptor stimulation. IL7 and IL7RA deficient mice have depleted thymocyte and T-lymphocyte levels [8].

CCR7 regulates dendrocyte and lymphocyte migration, both to and within lymph nodes. This migration is essential for communication between innate and adaptive immune systems. CCR7 is therefore required for the efficient induction and regulation

of adaptive immune responses. CCR7 is also involved in the development of antigen tolerance [9].

TLR4 is a pathogen recognition receptor of the innate immune system, expressed on pulmonary epithelial cells, and in abundance on pulmonary dendrocytes. TLR4 single nucleotide polymorphisms increase the risk of RSV disease [10]. Binding of RSV antigen to TLR4 initiates innate immune responses involving NF-κB mediated production of signaling and anti-viral factors; dendrocyte activation; granulocyte, macrophage and NK-cell recruitment; and dendrocyte migration to lymph nodes, where they initiate the adaptive immune response [4, 11]. TLR4 is also a low molecular weight hyaluronan receptor [12]. CD44 mediates immune responses initiated by hyaluronan/ TLR4 interactions [13], but its expression was not associated with predisposal to RSV infection.

IL7RA and CCR7 are not previously associated with RSV, but mice deficient in these proteins have a severe combined immunodeficiency phenotype [8, 9]. The clinical relevance of gene expression levels seen in this study is unknown. The children in our study did not have life-threatening disease, chronic disorders or repeat infections. The immune phenotype predisposing to RSV disease that is suggested by this study is therefore not likely to lead to severe immunodeficiency.

Cohort samples were taken after a stressful experience (birth), evoking an immune response, and we can therefore make conclusions on the immune response of these neonates. We previously reported downregulation of TNF receptor 25, Dicer and the NF-κB system in this cohort [5, 6]. In light of these and current findings, it seems reasonable to speculate that in children who develop RSV disease, immunological differences present at birth may involve initiation of the innate immune response,

communication between innate and adaptive immune systems, and homeostasis of peripheral T-lymphocytes. A unifying cause explaining all of our findings might be genetic or epigenetic variations in one or more of the genes we have described. Inter-group variation in the differential leukocyte count could also explain our findings, particularly for IL7R, which is likely expressed by lymphocytes, and TLR4, which is likely expressed by myeloid lineages. Unfortunately we did not perform such a count in our cohort. A recent study has described no differences in innate immune cell count at 1 month of age between infants with and infants without later RSV infection [14].

El Saleeby et al described a greater risk of severe disease in children with higher RSV nasal viral loads and delayed viral clearance [15]. Our findings may explain this association: an impaired immune response due to reduced activation of the innate immune response, and delayed communication between innate and adaptive immune systems could allow for greater viral replication, more extensive epithelial cell infection and a greater risk of pulmonary dissemination early during the course of infection, ultimately leading to greater RSV-mediated cell death, and therefore a more severe immune response when cells of the innate and adaptive immune systems have been adequately recruited.

Conclusion:

We find downregulation of *IL7R* and *CCR7* in the cord blood of children later testing positive for respiratory syncytial virus. In addition, those with severe disease had significant downregulation of *TLR4*. Downregulation of these leukocyte cell surface receptors may cause an impaired immune response to RSV, allowing greater viral replication and tissue damage, and thus more severe disease.

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Figure 1: Gene expression at birth in the cord blood of children who later test positive for RSV.

Note: Bars in panel A and B represent either [◊]the difference in the means, or [†]the Hodges-Lehman estimator for the difference between the medians of the control group and patient group for each gene, with 95% CI. **Panel A:** Gene regulation for 47 children with positive RSV tests compared to 17 controls. The dotted line represents the control group median or mean. There is significant downregulation of *IL7R* and *CCR7*. **Panel B:** Disease severity subgroup analysis. There is a significant ANOVA for *IL7R* and *CCR7*, and a tendency to significance for *TLR4*. Post-testing showed a significantly different control group compared to disease groups for *IL7R* and *CCR7*, indicating that downregulation of these genes predisposes to RSV disease, but does not affect the severity of disease. For *TLR4*, the severe disease group is significantly different from the control group *p = 0.003[◊], indicating that downregulation of *TLR4* is associated with severe RSV disease, but not mild disease. **Panel C:** Log ratios of target protein to CD45 in Western blots of white blood cells isolated from cord blood. Data is expressed as mean (95% CI). There are no significant differences between control and RSV disease groups.

[◊]Student's T-test; [†]Mann-Whitney test; [#]One-way ANOVA.

Table 1: Birth, clinical and intervention data for control, mild RSV and severe RSV groups

	CONTROL	MILD DISEASE	SEVERE DISEASE	Significance
Number	17	22	25	-
Male: Female ratio	8 : 9	9 : 13	14 : 11	p = 0.58 [1]
BIRTH	(n = 17)	(n = 18)*	(n = 24)*	
Vaginal	15 (88%)	13 (72%)	19 (79%)	p = 0.50 [1]
Instrumental	2 (12%)	2 (11%)	2 (8%)	p = 0.93 [1]
Caesarian Section	0 (0%)	3 (17%)	3 (13%)	p = 0.24 [1]
Mean Gestational Age (S.D.), weeks	39.6 (1.5)	39.2 (1.9)	39.2 (1.6)	p = 0.71 [2]
Mean Birth Weight	3674 (601)	4026 (810)	3575 (509)	p = 0.07 [2]

(S.D.), grams			
1 min Apgar < 8	0 (0%)	3 (17%)	2 (8%)	p = 0.21 [1]
5 min Apgar < 8	0 (0%)	1 (6%)	0 (0%)	p = 0.49 [1]
CLINICAL FEATURES:	(n = 14) †	(n = 25) †	
Median age RSV-positive (IQR), months	3 (2 - 10)	3 (1- 8)	p = 0.60 [3]
Significant Dyspnea◆	0 (0%)	21 (84%)	-
Apnea	0 (0%)	2 (%)	-
Cardiovascular Compromise	0 (0%)	3 (%)	-
Highest pCO2, mean (S.D.), kPa	5.74 (0.98)	6.49 (1.95)	p = 0.12 [4]
Lowest O_2 saturation◊; mean (S.D.), %	98 (2.3)	94 (5.6)	p = 0.005 [4]
Highest Respiratory Rate; Mean (S.D.), /min	40 (9.7)	50 (8.4)	p = 0.002 [4]
Pulse on admission; mean (S.D.), /min	160 (29)	155 (20)	p = 0.57 [4]
Weight on admission; mean (S.D.), grams	6783 (1825)	7719 (2952)	p = 0.33 [4]

Duration of symptoms on admission; mean (S.D.), days	4.2 (3.5)	3.6 (1.6)	p = 0.62 [4]
Length of stay; median (IQR), days	2 (2 - 4)	4 (3 - 5)	p = 0.002 [3]
INTERVENTIONS			
CPAP/ Ventilator	0 (0%)	0 (0%)	-
Supplemental Oxygen	0 (0%)	8 (32%)	-
Supplemental Fluids			
Intravenous	0 (0%)	2 (8%)	-
Feeding Tube	0 (0%)	3 (12%)	-

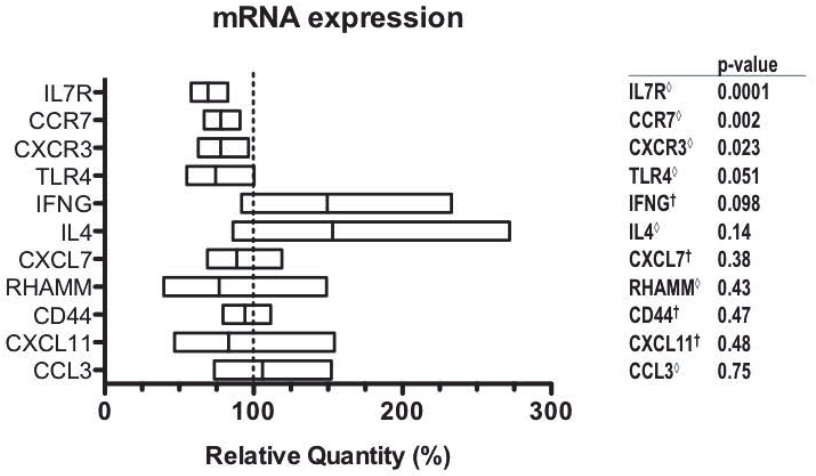
Note: There were no significant differences in gestational or delivery factors that might affect gene expression. Patients in mild and severe groups were ill at similar ages. There was a statistically significant reduction in oxygen saturation, increase in respiratory rate and longer length of stay in the severe disease group, and a tendency to higher pCO2. Clinical features and interventions incorporated in the algorithm for disease severity were not analyzed statistically. *Birth records were missing for 4 patients with mild, and one patient with severe disease. †Apart from age on RSV-positivity and gender, clinical features were unavailable for 8 RSV positive patients not referred to hospital, classified with mild

disease. ◆Pediatrician's assessment of respiratory effort. [◊]Lowest O_2 saturation irrespective of oxygen administration. pCO2: capillary partial pressure of carbon dioxide.

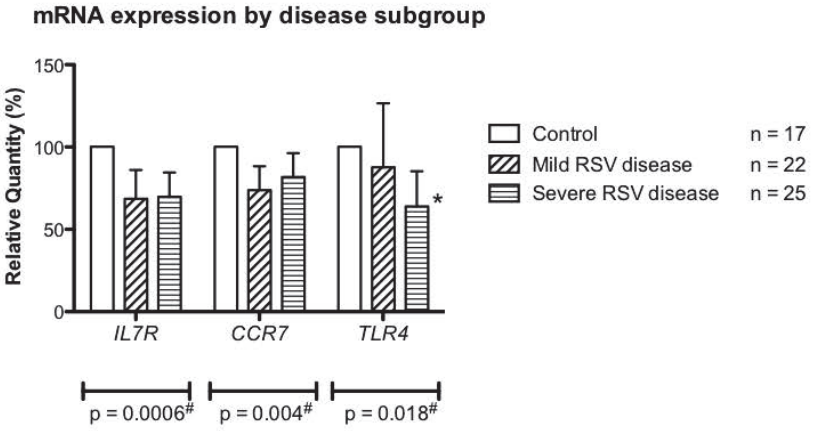
[1]Chi-square test comparing all 3 groups; [2]One-way ANOVA; [3]Mann-Whitney test; [4] Student's t-test

Gene expression at birth in children with confirmed RSV disease

A



B



C